STUDIES ON SYSTEMIC EFFECTS OF CYTOMEGALOVIRUS INFECTION AFTER RENAL TRANSPLANTATION

WILLEM J. VAN SON

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Stellingen

1. Bij iedere patient met een cytomegalovirusinfectie na niertransplantatie kan een gestoorde longfunctie worden aangetoond.

2. Echografisch onderzoek van de getransplanteerde nier dient bij voorkeur te geschieden door de transplantatie-nefroloog.

3. Vesicocalicostomie kan bij patienten met recidiverende postrenale problematiek van de getransplanteerde nier als laatste redmiddel met succes worden toegepast.

   (Van Son et al, J Urol 1986; 136: 889-891)

4. Een goed diermodel voor de humane diabetische nefropathie is vooralsnog niet voorhanden.

5. Niertransplantatie zonder voorafgaande chronische dialysebehandeling is een aantrekkelijk en verantwoord alternatief in de behandeling van patienten met terminale nierinsufficiëntie.

   (Tegzess et al, Trans Proc, vol XIX, 1, 1987; 2555-2556)

6. Bij de beschrijving van ziektebeelden moet het gebruik van de termen secundaire CMV infectie en reactivatie van een CMV infectie als misleidend beschouwd worden, gezien de recent verbeterde determineringstechnieken van het CMV.

7. Detectie van cytomegalovirus-antigenen in perifere bloedleucocyten is van groot belang voor de vroegdiagnostiek van een actieve CMV infectie na niertransplantatie.

8. Zoals transplantatie reincarnatie van een donororgaan is, zo is reincarnatie transplantatie van de ziel.

9. New York is an arrogant city; it has always wanted to be all things to all people, and in a surprising amount of time it has succeeded.

   (Paul Goldberger)

10. Indien een ernstig zieke patient opgenomen is zonder dat de diagnose snel duidelijk wordt dreigt polypragmasie ofte wel veroordeling tot T.P.R.: Totale Parenterale Ravage.

11. Het rijden op tubes i.p.v. op draadbandjes is in het geval van toerrijden snobistisch.

12. In verband met de toenemende afhankelijkheid van computers en tekstverwerkers in een modern ziekenhuis, ware het wenselijk dergelijke apparatuur aan te sluiten op een noodstroomvoorziening.

13. Bij de opmars van digitale technieken in de geneeskunde wordt soms vergeten dat digitaal ook vertaald zou kunnen worden in een aanbeveling voor het gebruik van vingers: een goed lichamelijk onderzoek.

14. De macrofaag is de pitbull terriër van het immuunsysteem.

Stellingen behorende bij het proefschrift 'Studies on systemic effects of cytomegalovirus infection after renal transplantation' door Willem J. van Son.

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RIJKSUNIVERSITEIT TE GRONINGEN

STUDIES ON SYSTEMIC EFFECTS OF CYTOMEGALOVIRUS INFECTION AFTER RENAL TRANSPLANTATION

PROEFSCHRIFT

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1987
DRUKKERIJ VAN DENDEREN B.V.
GRONINGEN

Eerste promotor : Prof. dr. T. H. The
Tweede promotor : Prof. dr. G. K. van der Hem
Derde promotor : Prof. dr. R. Peset Reig

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Prof. dr. J. D. Elema
Prof. dr. R. van Schilfgaarde

De firma Sandoz subsidieerde een deel van de drukkosten.

Dedicated to New York City

'If I don't manage to fly, someone else will.
The Spirit wants only that there be flying.
As for who happens to do it,
in that He has only a passing interest'.

Rilke

CONTENTS

VOORWOORD

Dit onderzoek werd verricht op de afdeling Niertransplantatie (hoofd: A. M. Tegzess) binnen de afdeling Nefrologie (hoofd: Prof. dr. G. K. van der Hem) in nauwe samenwerking met de afdelingen Klinische Immunologie (hoofd: Prof. dr. T. H. The), Longfunctie (hoofd: Prof. dr. R. Peset Reig) en de afdeling Immunochemie (hoofd Dr. J. Marrink) van de Kliniek voor Inwendige Geneeskunde (voorheen hoofd: Prof. dr. E. Mandema).

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CHAPTER 1

INTRODUCTION

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1.1. History and fundamental properties of the cytomegalovirus.

The term cytomegalovirus (CMV) was first used by Weller in 1960, because of the characteristic cytomegaly observed in CMV infected cells (1,2). Six years earlier in 1954 Smith and coworkers were able to isolate the virus for the first time from the salivary glands of mice in tissue culture (3). But probably the first description of the virus was given by Jesionek and coworkers in 1904 and by Löwenstein in 1907, who found cytomegalic cells in autopsic material from children (4,5). In the last 20 years an abundance of new information about the characteristics of this virus has emerged from new laboratory techniques, numerous studies in animal models and clinical studies in immunosuppressed patients. Biologically, CMV is a DNA virus belonging to the herpesvirus group that also includes varicella, herpes zoster, herpes simplex virus (HSV), and Epstein-Barr virus (EBV). It has a double stranded DNA core, an icosahedral symmetry of its capsid composed of 162 capsomeres and a surrounding envelope (6).

There are a number of different strains of CMV. All have specific characteristics, including a strong propensity for cell association and lability, a tendency to remain latent, and possibly a potential for malignancy (7). After a primary infection (patients with no previous CMV infection) the CMV remains dormant in a variety of cells throughout the body and is capable of being reactivated under certain conditions such as immunosuppression, allograft rejection and hormonal changes of pregnancy (8). Under these circumstances CMV emerges from its latent state and is capable of causing clinical disease again (secondary infections) (8). Cytomegalovirus is known to reside in splenic B cells, salivary gland tissue, the prostate, testes and probably macrophages and peripheral blood leucocytes (7,8). The transplanted kidney is also known as a possible source of latent CMV (9,10). Epidemiologically, CMV is endemic and has worldwide distribution. Seropositivity for CMV among normal populations varies considerably, the reported range being 28-100% (11). Its prevalence greatly depends on age, race, socio-economic and geographic factors; the Asian

and African countries being the area's with the highest rate of occurrence (11). In West-European countries, about 50% of the blood donor population between 18-65 years of age are seropositive, but it is estimated that only 1-12% of the donors are actually infectious (12).

1.2. **Clinical manifestations of CMV disease in renal transplant patients.**

Cytomegalovirus is the single most important infectious agent complicating the course of renal transplant recipients, and the incidence of CMV infections has been reported as high as 43-92% (13,14). Although many patients have asymptomatic CMV, most investigators agree that CMV has an impact on graft and patient survival (8,15,16). While this high incidence of CMV infection seems to be modified by the introduction of the immunosuppressive agent cyclosporine A (CsA) (17), CMV infection after renal transplantation still is an important factor influencing graft and patient survival especially when anti-thymocyte globulin (ATG) is used as additional immunosuppressive therapy (18).

From the patients that develop clinical manifestations of CMV infection, either due to primary or reactivation infection, more than 90% do so in the period 1-6 months posttransplant (8). During the first 1-6 months after transplantation 60% of the febrile episodes are due to CMV (16,19). When the patients with a CMV infection are symptomatic, the symptoms may vary greatly. Most of the patients have a so-called 'self-limiting' syndrome consisting of fever (often spiking), arthralgia, leucopenia and/or thrombopenia, and abnormalities in liver enzymes (SGOT, SGPT) (11). In this form CMV disease has characteristics of a systemic disease, since it is not known whether all of the symptoms are caused by the infectious virus itself or, alternatively, are mediated by 'factors' induced by the CMV infection. With tapering of the immunosuppression the great majority of the patients will have a complete recovery of the syndrome.

Renal involvement during CMV infection is frequently observed, but the cause of it is controversial (8). Simmons et al. suggested in 1970 for the first time, that CMV infection could be associated with an increased risk of allograft dysfunction and graft loss (20). They suggested the possibility that infection might trigger allograft rejection (20). Although disputed by many others (21) recent data suggest that a relationship between CMV infection and renal allograft dysfunction exists (8). Many investigators have, more recently, studied the possible relationship between CMV infection and allograft rejection. Betts and coworkers demonstrated CMV infection followed by graft rejection (22). In this respect it is noteworthy that Henny et al. demonstrated an increase in class II antigen expression during CMV infection on peripheral blood leucocytes and also

on renal tubular cells (23). This phenomenon may trigger, at least theoretically, allograft rejection. Others documented rejection followed by seroconversion and virus isolation (24,25). Although the exact sequence of events is still controversial, most data suggest that allograft rejection precedes active infection with CMV. So, rejection is probably important for the occurrence of primary infections or reactivation of latent virus (6), directly or indirectly via immunosuppression. Alternatively, in 1981, Richardson et al. (26) described a distinctive pattern of glomerular injury in renal allografts which they associated with CMV viremia without relation to allograft rejection. The pathological features consisted of diffuse endothelial hypertrophy and necrosis, accompanied by accumulation of fine fibrillar webs of periodic acid-Schiff (PAS)-positive material and mononuclear cells that resulted in obliteration of the glomerular capillaries (26). Furthermore, fibrin and IgM as well as C3 were found by immunofluorescence. No viral particles were detectable by electron microscopy or by immunofluorescence using monoclonal antibodies directed to CMV early and late antigens (26). Recently the existence of CMV glomerulopathy has been disputed (27). Herrera and coworkers state that the pathological condition that has been considered as cytomegalovirus glomerulopathy probably represents rejection, either a peculiar anti-endothelial type of rejection or a protracted, early, or partially resolved vascular type of rejection (27). It is noteworthy that patients without an allograft who contract CMV infection do not develop lesions as described by Richardson et al. (8,27).

Gastrointestinal symptoms during a CMV infection are numerous including gastrointestinal bleeding from ulcers (esophageal, gastric, coecal), gastritis and pancreatitis (16,28) as well as granulomatous hepatitis (29,30). Inclusion bodies (16) and sometimes vasculitis (31) are found at the site of the ulcers in the alimentary tract. Other manifestations of CMV infections posttransplant are protean: lymphadenopathy, rash, hepatosplenomegaly, conjunctivitis, pericarditis, myocarditis, Guillain Barré syndrome, vasculitis and chorioretinitis (8,13,32,33,34,35). Of the more common manifestions of CMV infection in the transplant patient, pneumonia is the one that separates serious illness from more benign disease (8,13,16). The most common form of CMV pneumonitis is a bilateral, symmetrical interstitial process that affects predominantly the lower lobes of the lungs (fig. 1). Alternatively, less frequently, a more lobar pattern may be found (fig. 2) and even a solitary pulmonary nodule has been reported solely due to CMV (36). Peterson et al. analyzed the risk factors in the development of cytomegalovirus-related pneumonia in renal transplant recipients (37). They found that recipients of kidneys from seropositive donors had a more than threefold greater risk of developing CMV related pneumonia as compared to recipients of kidneys from seronegative donors (37). Furthermore an increased risk was found in patients who received ATG as immunosuppressive therapy

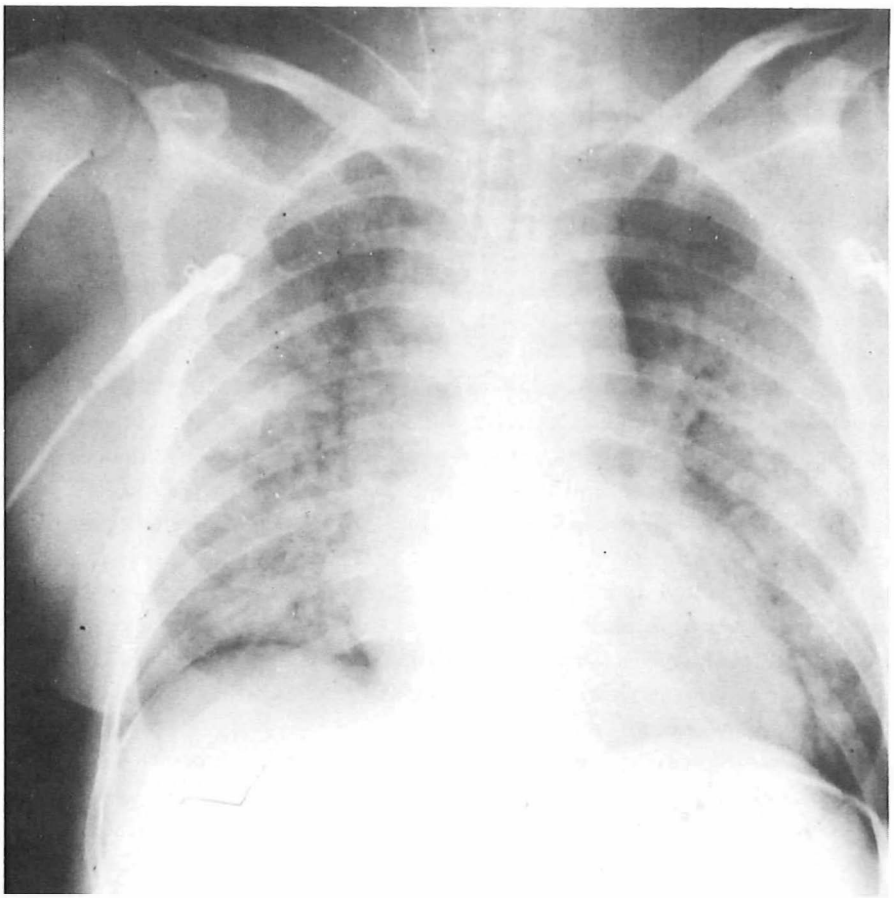

Figure 1.

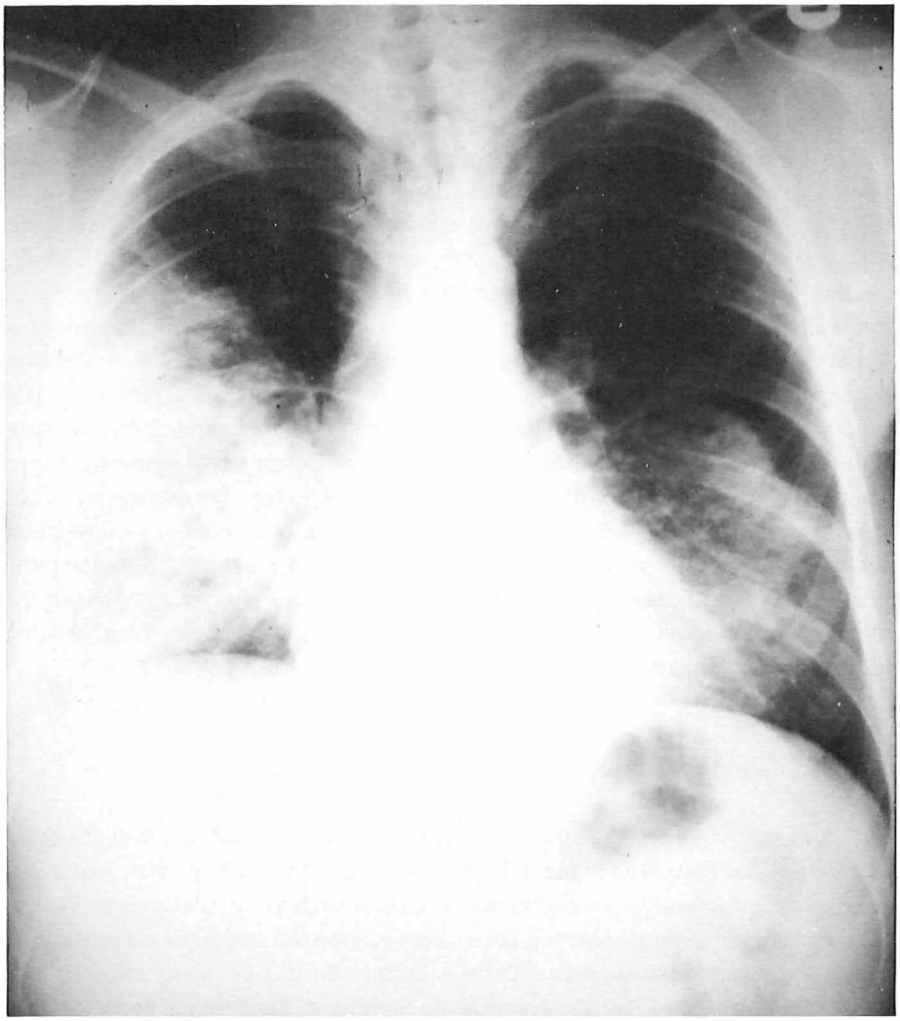

Figure 2.

(37). Although the exact pathogenesis of the CMV pneumonitis is not known (38), some investigators suggest that it could be an immunologically mediated phenomenon rather than the result of direct viral damage to the lung (39). Clinically, patients with CMV pneumonia present with a dry, non productive cough followed a few days later with dyspnea and tachypnea eventually leading to respiratory failure (8). Essentially the course of the disease is slow and an acute rapid deterioration of the dyspnea and other symptoms must alert the clinician to the possibility of superinfection with for instance Pneumocystis Carinii, Aspergillus fumigatus or gram negative bacilli (8).

Finally, in 1977 Simmons and coworkers described the characteristics of a lethal CMV syndrome ('wasting disease'), in which patients suffer an unrelenting disseminating disease ending in death despite cessation of the immunosuppression (40). The clinical characteristics of the lethal CMV infection following renal transplantation are prostration, orthostatic hypotension, severe pulmonary dysfunction with undersaturation, hepatic dysfunction, muscle wasting, central nervous system depression, and severe gastrointestinal symptoms associated with bleeding from ulcers, ultimately leading to death (40). Fortunately, with the modern immunosuppressive regimens and improved diagnostic techniques for early detection of CMV infections, the incidence of this lethal syndrome has become less frequent (8).

1.3. **CMV and Immunity.**

Cytomegalovirus and the immune system have reciprocal effects on each other. The host factors involved in the defence against the CMV are: genetic make-up, non-specific immunity, specific humoral factors such as circulating antibodies, specific cellular immune factors, for example cytotoxic and helper T cells and, indirectly, the immunosuppressive drugs used (section 1.2). So far, in man, the possible additional role of the complement system in the defence against CMV has not been studied thoroughly.

As to the genetic factors involved, most evidence for genetic controlling of susceptibility and resistance to CMV has emerged from animal studies. Diosi reported evidence for genetic transmission of susceptibility or resistance to murine CMV (MCMV) (41). Using Swiss-strain mice and wild strain mice, they found that the wild strain inherited susceptibility to MCMV as an autosomal dominant trait and resistance as an autosomal recessive trait, controlled by a single pair of genes (6,41).

Chalmer and Grundy confirmed, also in the murine model, these observations as they also found that susceptibility or resistance to the virus was controlled by genes of the H2 complex (42,43). In man, an association between HLA-DR

antigens with cellular immune response to infectious agents was found (44), and recently Roenhorst et al. (45) demonstrated an increased incidence of active cytomegalovirus infection in renal transplant recipients positive for HLA-DRw6. Furthermore, they found that recipients positive for HLA-DRw6 with secondary infection excreted infectious virus more often and also were more symptomatic than HLA-DRw6 negative recipients (45).

Humoral immunity as a host defence to CMV is, as it exerts its effect outside the cell, probably relatively ineffective, since CMV is a strongly cell associated virus and usually remains inside the infected host cell. But, virus induced neo-antigens expressed on the surface of CMV infected cells (CMV membrane antigens: CMV-MA) may serve as primary target for host immunological humoral as well as cellular defence (46). Recently, Middeldorp found evidence for the importance of humoral immunity directed against CMV-MA, since he found that the appearance of antibodies to CMV-MA was related to subsequent recovery from CMV disease (47). The cellular host defence probably also plays a pivotal role in the defence against CMV (6,8). Cytotoxic T lymphocytes as well as natural killer cells and antibody dependent cell mediated cytotoxic cells are found during CMV infection. Of the non virus-specific factors involved in the defence to CMV, macrophages are believed to play an important role (6). The counterpart in the mutual relationship between CMV and the immune system is formed by the strong effects exerted by CMV on host immunity. Cytomegalovirus infection is an important contributor to the net state of immunosuppression present in a given renal transplant patient (6,8,48). The immunosuppressive effect of the virus makes the patient prone to superinfection with opportunistic pathogens (21,49,50). Patients with a CMV infection have a marked decrease in cellular immunity, for instance an impaired response to skin testing to Candida, mumps and tuberculine antigens (8). During CMV infection an inverted ratio of T4 positive and T8 positive cells (T4/T8 ratio) is found due to depression of the number of T4 positive cells (helper cells) and an increase in the number of T8 positive cells (suppressor cells) (8). Cytomegalovirus infection is also associated with suppressed monocyte function and monocyte-induced suppression of lymphocyte function (51). The additional role of the leucopenia which is so frequently observed during infection in the net state of immunosuppression remains unclear, although severe leucopenia during symptomatic CMV disease lasting for more than 5 days has been associated with high mortality due to superinfection (49). The impaired cellular immunity found during a CMV infection is not confined to the period when the patient suffers from CMV disease. Recently it has been shown that patients with a secondary CMV infection have a long lasting negative effect on immunity against alloantigens and CMV infected targets (52).

1.4. **Purpose of this thesis.**

The purpose of the studies described in this thesis was to gain insight into the mechanisms that lead to systemic effects of CMV infection after renal transplantation. One of those systemic manifestations is described in *chapter 2*, where we present a possibly new entity of the CMV-syndrome. In *chapter 3* the possible role of the complement system in the defence against CMV in renal transplant recipients is presented. In *chapter 4*, as a consequence of the study presented in chapter 3, we tried to elucidate whether complement activation during CMV infection is due to classical pathway activation or alternative pathway activation of complement. A longitudinal study is presented on the presence of complement breakdown products and biologically active peptides that are formed during complement activation as well as studies on the alternative pathway of complement. Furthermore studies are presented on the presence of circulating immune complex-like material, since circulating immune complexes are known to activate the classical pathway of complement. In *chapter 5* pulmonary involvement during CMV infection is studied. A sensitive method for detecting early pulmonary involvement during CMV infection is presented. The aim was to detect early pulmonary involvement before there are chest roentgenographic abnormalities and before there are pulmonary symptoms. In *chapter 6* early pulmonary involvement was further investigated. If there is early pulmonary involvement in patients with a CMV infection, even if they have no symptoms, could it be that this phenomenon is related to the complement activation found during infection? The rationale behind this possible relationship is the possible analogy with complement induced pulmonary dysfunction found during hemodialysis (53) and in respiratory distress syndrome (54,55). Finally, in *chapter 7* we discuss the relevance and causal relationship between signs and symptoms and complement activation found during active CMV infection after renal transplantation. Our studies might give new insights to our knowledge of systemic effects of viral infections in general.

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CHAPTER 2

PNEUMATOSIS INTESTINALIS IN PATIENTS AFTER CADAVERIC KIDNEY TRANSPLANTATION: POSSIBLE RELATIONSHIP WITH AN ACTIVE CYTOMEGALOVIRUS INFECTION

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PNEUMATOSIS INTESTINALIS IN PATIENTS AFTER CADAVERIC KIDNEY TRANSPLANTATION: POSSIBLE RELATIONSHIP WITH AN ACTIVE CYTOMEGALOVIRUS INFECTION

Willem J. van Son, Eric J. van der Jagt*, Fokko J. van der Woude, Maarten J.H. Slooff**, Sijtze Meijer, T. Hauw The, Adam M. Tegzess, Leendert B. van der Slikke and Ab J.M. Donker.

From the Renal Transplantation Unit, Departments of Internal Medicine, Radiology* and Surgery**, University Hospital Groningen, The Netherlands.

SUMMARY

Four patients are described with pneumatosis intestinalis following cadaveric kidney transplantation, all with severe cytomegalovirus (CMV) infection. Two patients had a primary infection and 2 patients had a reactivation of CMV. One patient died because of disseminated CMV infection. Multiple inclusion bodies were found at postmortem examination in lungs, liver and at the site of the ulcers in the gastrointestinal tract. Two patients had concomitantly an active, non-obstructive duodenal ulcer.

In a control population of 17 patients who suffered from a duodenal ulcer post transplant without any evidence of CMV infection, we could not demonstrate pneumatosis intestinalis. We suggest a possible causal relationship between pneumatosis intestinalis and active CMV infection. The mechanisms that could be responsible for this relationship are discussed.

INTRODUCTION

Gastrointestinal complications after cadaveric kidney transplantation are numerous and include esophagitis, ulcers with or without gastrointestinal hemorrhage or perforation, pancreatitis, and infarction (1,2). These complications are believed to be related to the use of immunosuppressive drugs, especially corticosteroids (1,2,3). Pneumatosis intestinalis is an uncommon disease the pathogenesis of which is still under discussion. Reports of

pneumatosis intestinalis in patients with a kidney transplant are rare and are also believed to be related to the use of immunosuppressive drugs (1,3).

We recently observed 2 patients with a cadaveric kidney transplant and pneumatosis intestinalis. Both patients had an active CMV infection. The association prompted us to look in a retrospective way at our transplant patient population to determine whether we could find other patients with pneumatosis intestinalis in combination with an active CMV infection.

MATERIALS AND METHODS

In 1982 we studied 2 patients who suffered from pneumatosis intestinalis. Retrospectively we found in our transplant population 2 other patients with this abnormality. Patient data are given in Table I. Case histories are reported in the Results section. All 4 patients had received a cadaveric kidney transplant and were treated with standard immunosuppression consisting of azathioprine and corticosteroids only. All 4 patients had an active CMV infection with fever, arthralgia, leucopenia, thrombocytopenia, liver function abnormalities and changes in CMV serology, when pneumatosis intestinalis was detected. The diagnosis of pneumatosis was made on plain roentgenogram of the abdomen with the patient in supine position, when intramural gas was present (4,5). The serological diagnosis of an active CMV infection was defined as a fourfold or greater rise in titers of complement fixing antibodies (CFA) against CMV or a fourfold or greater rise in antibodies to CMV early (EA) and late (LA) antigens. Antibodies to CMV early (EA) and late (LA) antigens were detected by indirect immunofluorescence, a technique described elsewhere (6). The patients were considered to be seronegative for CMV when the CFA titers were less than 1:4 and the titers of antibodies to CMV-EA and -LA less than 1:40.

Control population

Two out of our 4 patients with pneumatosis also had an active, non-obstructive duodenal ulcer (Table I) at the time when the diagnosis of pneumatosis was made. According to the literature gastric or duodenal ulcers (when obstructive) can cause pneumatosis (7,8,9). Therefore we restudied all abdominal roentgenograms of patients within our transplant population who had a gastric or duodenal ulcer in the period from 1971-1982. The diagnosis of ulcer had been made by upper gastrointestinal series or by endoscopy. An active CMV infection could be excluded in these patients at the time when the ulcer was diagnosed. These patients also received standard immunosuppression (azathioprine and

Table I. Clinical details of the 4 patients with pneumatosis intestinalis.
M = male, F = female

Patient no.	age/sex	type of CMV-infection	gastro-intestinal symp. before Tx	diagnosis of pneumatosis weeks after Tx	roentgeno-graphic diagnosis	pneumo-peritoneum	concomittant ulcer	final outcome
1	47/F	primary	none	8	pneumatosis intestinalis cystoides (jejenum + ileum)	—	yes (UD)	complete recovery; good renal function
2	31/F	reactivation	none	8	pneumatosis intestinalis linear type + cystoides (ileum + colon)	+	no	complete recovery; marginal renal function (vascular rejection)
3	49/F	primary	none	6	pneumatosis intestinalis linear type (jejunum + ileum)	+	no	complete recovery; good renal function
4	22/M	reactivation	none	8	pneumatosis intestinalis linear type ileum	—	yes (UD)	died; generalized CMV-infection (liver, gut, lungs)

corticosteroids). In 18 out of the 382 patients transplanted between 1971-1983 a gastric or duodenal ulcer had been proved. In 17 cases we had sufficient roentgenographic material to determine whether pneumatosis intestinalis had been present at the time the ulcer was found.

RESULTS

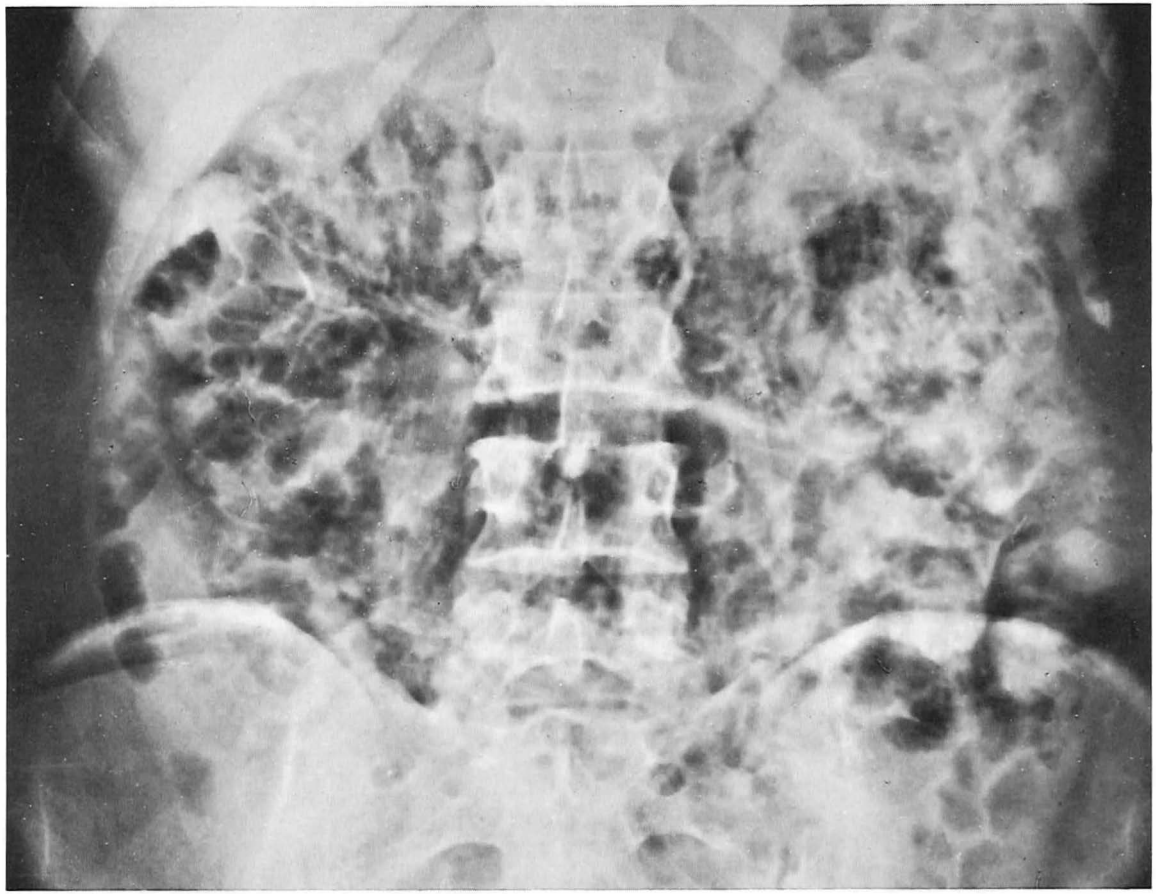
Case histories

Patient number 1

A 47 year old woman received a cadaveric kidney transplant on 11-27-1982. After an uneventful start, an anti-rejection course was given at the 12th day post-operatively (p.o.) by augmentation of the oral prednisolone dose. Renal function improved rapidly. On the 31th day she complained of pain in the lower thoracoabdominal region. A gastroscopy revealed an ulcer in the lower esophagus and an active duodenitis. Cimetidine therapy was started and the immunosuppressive therapy was rapidly tapered. Because of thrombocytopenia the azathioprine medication was stopped for six days. On the 35th day p.o. serum creatinine began to rise while the body temperature rose to 38°C. On examination her kidney was swollen, hard and painful. An ultrasound examination disclosed no ureteral obstruction. Azathioprine medication was reinstituted and a second anti-rejection course was started on the 40th day. Renal function did not improve. On the 50th day patient suffered from severe arthralgia. On the 54th day she complained of abdominal pain localized around the umbilicus. A plain roentgenogram of the abdomen revealed pneumatosis intestinalis cystoides (fig. 1). No pneumoperitoneum was found. Laboratory investigation revealed leuco- and thrombocytopenia, and elevated liver enzymes (SGOT, SGPT). Seroconversion for CMV was found and the diagnosis active CMV infection was made. A chest X-ray appeared to be normal. Azathioprine was stopped again and corticosteroid therapy was rapidly tapered. Oxygen therapy via Ventimask® (6 l/min) and feeding via nasogastric tube was started. Body temperature rose as high as 40°C and severe arthralgia persisted. On the 60th day the clinical situation improved as well as the renal function. On the roentgenogram of the abdomen the signs of pneumatosis intestinalis became less evident. Because of persisting abdominal pain a second gastroscopy was performed on the 68th day; it revealed an active, non-obstructive duodenal ulcer. A milkdrip was instituted and cimetidine therapy continued, with rapid improvement of the symptoms. The further course was uneventful and patient was discharged from the hospital on the 87th day with good renal function. The roentgenographic signs of pneumatosis intestinalis had completely resolved.

Figure 1.

Pneumatosis intestinalis, predominantly of cystic type (patient no. 1).



Patient number 2

A 31 year old woman received a cadaveric kidney transplant on 12-09-1982. On the fifth postoperative day an anti-rejection course was instituted because of acute rejection, followed by rapid improvement of renal function. On the 29th day p.o. renal function rapidly deteriorated. An ultrasound examination of the graft showed no abnormalities. Antibodies to CMV-EA and -LA did not increase, probably because the titers were already high before transplantation. A second anti-rejection course was given with only slow improvement in renal function. On the 57th day p.o. she complained of abdominal discomfort localized around the umbilicus. No abnormalities were found on physical examination. Body temperature rose and she complained of arthralgia. Laboratory examination disclosed leucopenia, elevated liver enzymes (SGOT, SGPT) and a rise in serum creatinine. At that moment a significant rise in titers of CFA to CMV was found. The diagnosis secondary CMV infection (reactivation) was made and the azathioprine medication was withdrawn while the prednisolone dose was rapidly tapered. A plain roentgenogram of the abdomen disclosed pneumatosis intestinalis (linear type and cystoides) of the ileum and colon, complicated by pneumoperitoneum. Oxygen therapy via Ventimask® and feeding via nasogastric tube were started. The clinical status rapidly improved and all signs of pneumatosis intestinalis disappeared. In contrast, however, was a slow progression in renal failure. Because of the active CMV infection and the abdominal complications the situation was accepted and patient was discharged with a creatinine clearance of 10 ml/min.

Patient number 3

A 49 year old woman received a cadaveric graft on 04-01-1981. After an uneventful start, an anti-rejection course was given on the 11th day p.o. Rapid improvement in kidney function followed. On the 33rd day p.o. patient complained of severe arthralgia. No abnormalities were found on physical examination, and there were only slight leucopenia and abnormalities in liver enzymes on laboratory examination. No seroconversion for CMV was found. Arthralgia persisted and on the 42nd day p.o. body temperature rose. Laboratory examination disclosed leucopenia, elevated liver enzymes (SGOT, SGPT) and slight deterioration in renal function. A chest roentgenogram surprisingly disclosed free air below the diaphragm! She had no abdominal symptoms whatsoever and no abnormalities could be found on physical examination. Liver dullness was present. Upper gastrointestinal series and a roentgenogram of the colon were negative. At that time seroconversion for CMV antibodies to EA and

LA was found. Azathioprine was withdrawn and the dose of prednisolone was tapered. Parenteral feeding was started. Free air below the diaphragm rapidly disappeared and parenteral feeding was stopped on the 50th day p.o. She was discharged on the 61st day with good renal function. Retrospectively, we could find a clear example of 'linear type' pneumatosis of the ileum and jejunum on the intravenous urogram, made at the time she started to complain of arthralgia (fig. 2).

Patient number 4

A 22 year old man received a cadaveric graft on 10-26-1972. He was seronegative at the time of transplantation (CFA to CMV). On the 8th day p.o. an anti-rejection course was started because of severe rejection. On the 11th day the graft was removed because of irreversible rejection, and a second transplant was performed immediately thereafter because a HLA compatible kidney was available. After an uneventful start an anti-rejection course was given on the 6th day p.o. resulting in improvement of renal function. On the 20th day the patient complained of pain in the epigastrium, and a duodenal ulcer was found on upper gastrointestinal series. The prednisolone dose was tapered. On the 24th day body temperature rose to 40°C and patient complained of severe arthralgia and abdominal discomfort. Leucopenia and elevated liver enzymes (SGOT, SGPT) were found on laboratory examination. All cultures (blood, urine) were negative, but the fever persisted. On the 38th day a gastrectomy according to Billroth II was performed because of severe hematemesis and melena. An active duodenal ulcer was found penetrating the pancreas. The clinical situation worsened with persisting spiking fever and deterioration in liver function. A re-resection and vagotomy were performed on the 47th day because of rebleeding. Multiple ulcers were found with no tendency to heal. The clinical situation worsened and the patient died on the 50th day p.o. in profound shock. No seroconversion in CFA to CMV could be demonstrated during the whole period of hospitalisation of this patient, although CMV had been isolated from the urine. Postmortem examination revealed disseminated infection with CMV with multiple inclusion bodies in lungs and liver. In the stomach and jejunum many ulcers were found that were considered responsible for the gastrointestinal bleeding. Inclusion bodies were found adjacent to the vessels in the ulcers (fig. 3). CMV virus was cultured from these sites. We could not demonstrate vasculitis at the site of the ulcers in the material available for re-examination at the time we performed our retrospective study. However, we could clearly demonstrate a linear form of pneumatosis intestinalis on the upper gastrointestinal series performed at the time patient suffered from spiking fever and arthralgia. Retrospectively, we also

Figure 2. Patient no. 3: pneumatosis intestinalis, linear type.

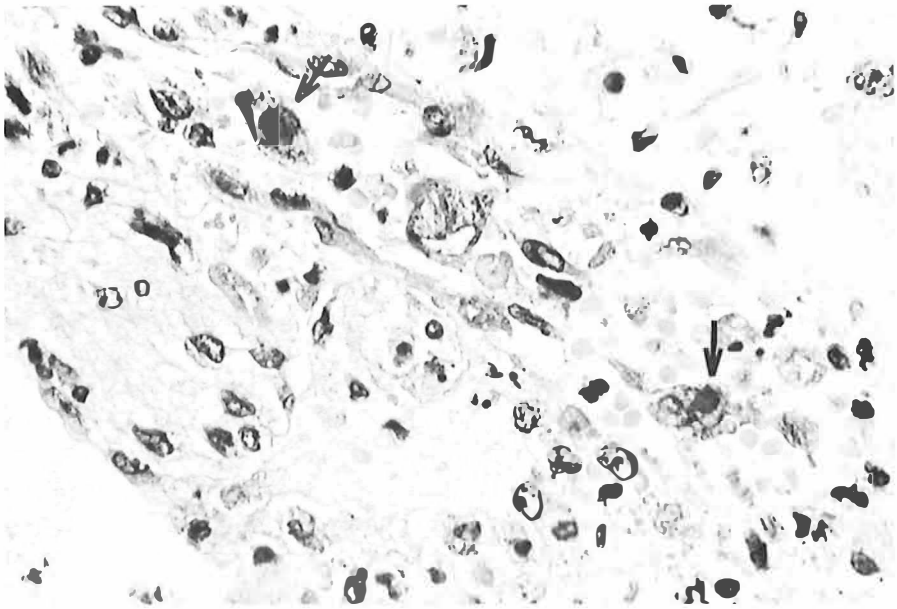


Figure 3. CMV-inclusion bodies (-->) at the site of an ulcer in patient no. 4.

re-examined serum samples of this patient with the indirect immunofluorescence technique for antibodies to CMV-EA and -LA (a technique not available in 1972). We found that this patient was seropositive at the time of transplantation, and furthermore, that a significant rise could be demonstrated in antibody titers to CMV-LA in the week before death.

Control population

In none of the 17 patients with a proven ulcer we could find any evidence of pneumatosis intestinalis at the time the diagnosis of ulcer was established.

DISCUSSION

Cytomegalovirus (CMV) is an ubiquitous agent and CMV infection is common in human populations throughout the world. The majority of the cases are however, subclinical (10,11). Clinical evident CMV infections are more common in patients during immunosuppression induced by drugs or by disease (12). The immunosuppressive regimens used in renal transplantation make renal transplant recipients particularly prone to CMV infections (11,13). Most of these infections

represent reactivation of latent virus (secondary infections), while the remaining cases are primary infections, some of which are believed to be transmitted from donor to recipient (11,13,14).

The incidence of active CMV infection (defined as a 4-fold or greater rise in titers of antibodies to CMV, or recovery of infective virions from any site) was said to be as high as 43-92% in renal transplant patients, as reviewed by Glenn (13). Most of the cases are subclinical and when the CMV infection is clinical, the symptoms may show a great variety. The so-called 'self limited syndrome', in about 40-50% of the patients infected (13) is well known. The self limited syndrome usually occurs between the 30th and 90th postoperative days and consists of prolonged, often spiking fever, arthralgia, leucopenia, abnormalities in liver enzymes, respiratory symptoms or infiltrates on chest roentgenogram, and impairment in renal function (13). Richardson and coworkers described a glomerulopathy associated with CMV viremia in renal transplant recipients (15). From 2 to 3 percent of the infected patients suffer an unrelenting disseminated disease ending in death despite cessation of the immunosuppression (13). The clinical characteristics of the lethal CMV infection following renal transplantation are prostation, orthostatic hypotension, severe pulmonary dysfunction with undersaturation, hepatic dysfunction, muscle wasting, central nervous system depression, and severe gastrointestinal symptoms associated with bleeding from ulcers, ultimately leading to death (16). Other manifestations of CMV infections post-transplant are protean: lymphadenopathy, rash, hepatosplenomegaly, conjunctivitis, Guillain-Barré syndrome and vasculitis (13,17).

CMV has been noted to reside in alimentary tract ulcers (13,18), sometimes associated with vasculitis at the site of the ulcer (18). Franzin and coworkers found evidence of CMV inclusion bodies in biopsies collected from gastroduodenal mucosa of patients with a renal transplant in 9 out of 20 patients. The presence of these CMV inclusion bodies was unrelated to viremia-induced or gastrointestinal symptoms at the time of the endoscopy (19). Most of the patients with evidence of these CMV inclusion bodies had been seronegative for CMV before transplantation (19).

Pneumatosis intestinalis is an uncommon disease the pathogenesis of which is still being discussed (8,9). This pathological condition consists of intramural gas collection, and can manifest itselves in two ways, depending on localization in the intestinal tract (4,5). The linear form of pneumatosis is seen when subserosal gas spreads longitudinally parallel to the lumen of the bowel. Radiologically, translucencies parallel to and just outside the gas filled bowel lumen are found (4,5). The submucosal form of intestinal pneumatosis leads to gas filled cysts. These cysts can also be found when the gas is subserosal. This form is designated pneumatosis intestinalis cystoides (4,5). As a result of bursting of the cysts into

the peritoneal cavity, a pneumoperitoneum can develop and very characteristically this pneumoperitoneum is asymptomatic because no peritonitis results from the sterile gas.

As to the pathogenesis of pneumatosis, the condition is often associated with intestinal obstruction, especially with pyloric obstruction caused by a peptic ulcer (20). Associations also have been made with chronic pulmonary disease (21), collagen diseases associated with vasculitis (systemic lupus erythematosus, periarteritis nodosa), or motility disorders of the bowel leading to (functional) obstruction (scleroderma) (22,23,24). Less frequently pneumatosis has been found in association with gastrointestinal infections with gas forming bacilla (Clostridium) (24), endoscopy (24), ischemic bowel disease (24), lymphoma (25), and leukemia (26). Reports of pneumatosis intestinalis in renal transplant patients (2 cases) (1,3) or liver transplant patients (2 cases) (27) are very rare and are believed to be associated with the immunosuppressive drugs used (1,3,27). Very recently the condition was described in 3 patients following allogenic bone marrow transplantation (28). Although the condition, according to the authors, was related to the immunosuppressive regimens and/or severe graft versus host disease, it is interesting that 2 of the 3 patients also suffered from severe CMV infection (CMV esophagitis, CMV pneumonia) (28).

We have described 4 patients with pneumatosis intestinalis after renal transplantation, all with concomitant severe CMV infection (2 primary infection, 2 secondary infection). Three patients had aspecific gastrointestinal symptoms at the time the diagnosis pneumatosis was made. In one case the finding was accidental because an intravenous urogram was made at the time when this patient had an active CMV infection. No abdominal symptoms whatsoever existed in this patient (case no. 3). In patient no 4, the CMV virus was also cultured from the ulcers of the intestinal tract. Because 2 of the 4 patients also had a non-obstructive gastric ulcer (possible related to the CMV infection), we retrospectively investigated all patients in our transplant population from 1971-1982 with a gastric ulcer to find evidence of pneumatosis intestinalis at the time the diagnosis of ulcus was made. We could not find any evidence of pneumatosis intestinalis in this control population. It was not possible to perform a similar study in patients with an active CMV infection without an ulcer, because of the lack of plain roentgenographs made at the time of infection. As to the pathogenesis of the pneumatosis in our 4 patients, one can only speculate. The negative findings in the control group suggest that the ulcers per se were not the cause of the condition. None of the patients had chronic pulmonary disease, gastrointestinal infections with gas forming bacilla, evidence of ischemic bowel disease, or a systemic disease like lupus erythematosus. The immunosuppression per se also seems unlikely as sole pathogenetic explanation, because of the low incidence of the condition in the whole transplant patient population. The

striking coincidence with a severe CMV infection in all 4 patients suggests that infection with CMV might be related to the pneumatosis. The mechanisms are still unclear, although possible explanations could be the primary cytopathogenic effect of CMV on the intestinal mucosa or vasculitis resulting from the CMV infection. As mentioned before, CMV is often found in the gastrointestinal mucosa with or without ulcers (13,18,19), and with or without vasculitis (17,18). The possible additional role of immunosuppressive drugs remains unclear.

In conclusion, we believe that pneumatosis intestinalis in a renal transplant patient might be associated with an active CMV infection, although the exact mechanism still must be elucidated. The reason that pneumatosis intestinalis in renal transplant patients is so infrequently found might be that plain roentgenograms of the abdomen are seldom made during an active CMV infection because the condition of pneumatosis can be asymptomatic. We suggest that roentgenographic examination of the abdomen should be performed in renal transplant patients during an active CMV infection, especially if (aspecific) abdominal symptoms are present.

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CHAPTER 3

COMPLEMENT ACTIVATION ASSOCIATED WITH ACTIVE CYTOMEGALOVIRUS INFECTION IN RENAL TRANSPLANT PATIENTS AND ITS ABSENCE IN TRANSPLANT REJECTION EPISODES

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Willem J. van Son, Fokko J. van der Woude, Gjalt K. van der Hem, T. Hauw The*, Theo Ockhuizen**, Adam M. Tegzess.

Renal Transplantation Unit, Departments of Internal Medicine, Clinical Immunology* and Immunochemistry**, University Hospital Groningen, The Netherlands.

SUMMARY

In 20 patients with a cadaveric renal allograft serial measurements were made of the serum complement factors C3, C4, factor B (FB) and C3d, the stable conversion product of C3. Measurements were started immediately before transplantation and continued thereafter once a week in order to investigate whether these assays help to differentiate between acute allograft rejection (R) and an active cytomegalovirus (CMV) infection. Fifteen patients had one or more R episodes, and 9 patients suffered from an active CMV infection. Six patients had an R episode and subsequently an active CMV infection 13-64 days after R. No significant changes were found in the levels of C3, C4 and FB during R or CMV infection. C3d levels remained unchanged or decreased slightly during R. However, there was a 43-500% increase in C3d level during CMV infection. This difference in the behaviour of levels of C3d during R and CMV infection is significant ($p < 0.01$), and suggests that serial measurements of C3d may be useful in differentiating CMV infection from R after renal transplantation.

INTRODUCTION

The incidence of active cytomegalovirus (CMV) infection has been reported as high as 43-92% in renal transplant patients, it is most likely caused by the immunosuppression used in these patients (1). To differentiate between an active

CMV infection and acute allograft rejection (R) can be very difficult because fever, a rise in serum creatinine, and a swollen painful kidney, as well as abnormalities in graft biopsies may also be found during a CMV infection (1,2).

Results from experimental as well as clinical studies suggest that the integrity of the complement (C) system is important for the defence against micro-organisms and viruses (3-6), but there is some doubt whether C is involved in the R process and whether changes in serum C levels during R might have diagnostic significance (7). On the other hand, CMV infection may induce immune complex disease (8). IgM immune complex-like material can be found in the circulation of renal transplant recipients during a CMV infection (8) which could activate the classical pathway of C. Only one study suggests a role for the C-system during CMV infection in renal transplant patients (9). Activation of the C-system can be evaluated quantitatively by measurements of C3d levels, the stable conversion product of C3 (10-12). For example, C3d levels are reported to be increased e.g. in active systemic lupus and rheumatoid arthritis (13,14).

We studied 20 renal transplant patients in a prospective way by serial quantitative measurements of C4 (classical pathway), factor B (FB, alternative pathway) and C3 (central common C-factor) and C3d, in order to investigate whether C is activated during an active CMV infection and whether this can help to differentiate between R and CMV infection.

MATERIALS AND METHODS

Twenty patients were studied. Each patient received a cadaveric transplant. Clinical details are given in Table I. Serum samples for determination of factor C3, C4, FB and C3d were drawn immediately before transplantation and at weekly intervals after transplantation. When R or CMV infection was suspected the measurements were made twice a week. Rejection was defined as an increase in serum creatinine of 20% or more together with one or more of the following: a swollen painful kidney; fever; decrease in urinary output; salt retention; and findings compatible with rejection in a renal biopsy (10 patients), in absence of postrenal abnormalities. Anti-rejection treatment consisted of augmentation of oral prednisolone dosage (prednisolone-azathioprine group) or a bolus of 1000 mg methylprednisolone given three times i.v. on successive days (Cyclosporin group). Active CMV infection was defined as a fourfold or greater rise in antibodies to CMV-early (EA) and -late (LA) antigens. Antibodies to CMV-EA and -LA (IgG and IgM) were detected by indirect immunofluorescence, a technique described previously (15). The patients were considered to be seronegative for CMV when complement fixing antibodies (CFA) titers were less than 1:4 and the titers of antibodies to CMV-EA and -LA less than 1:40. All

Table I. Clinical data of the study population

Pat.no.	Age	Sex	Original disease	Rejection	CMV	Mode of immunosuppression
1	29	F	reflux nephropathy	+ day 7	–	prednisolone/azathioprine
2	21	F	medullaric cystic disease	+ day 4	–	prednisolone/azathioprine
3	39	F	poststreptococcal GN	+ day 17	–	cyclosporine
4	22	M	proliferative GN	+ day 10	+ day 50, P	prednisolone/azathioprine
5	47	F	reflux nephropathy	+ day 10	+ day 59, P	prednisolone/azathioprine
6	31	F	chronic GN	+ day 4	+ day 68, R	prednisolone/azathioprine
7	42	M	hypoplastic kidneys	–	+ day 40, R	prednisolone/azathioprine
8	33	F	end stage kidney e.c.i.	+ day 5	–	cyclosporine
9	40	M	malignant hypertension nephrosclerosis	+ day 9	–	cyclosporine
10	53	F	nephrosclerosis	+ day 3	–	cyclosporine
11	53	M	diabetic nephropathy	+ day 7	–	prednisolone/azathioprine
12	35	F	chronic GN, nephrotic syndrome	–	+ day 29, P	cyclosporine
13	28	M	membranoproliferative GN	+ day 27	+ day 40, R	cyclosporine
14	51	F	focal glomerulosclerosis	–	+ day 50, P	cyclosporine
15	55	F	chronic GN	+ day 4 and 30	+ day 60, R	cyclosporine
16	39	F	membranoproliferative GN	+ day 8	+ day 62, R	prednisolone/azathioprine
17	33	F	amyloidosis	+ day 15	–	prednisolone/azathioprine
18	53	F	analgetics abuse	+ day 5	–	prednisolone/azathioprine
19	30	M	reflux nephropathy	–	–	prednisolone/azathioprine
20	38	F	polycystic disease	–	–	prednisolone/azathioprine

F = female, M = male, GN = glomerulonephritis, P = primary, R = reactivation

patients with a serological active CMV infection had one or more of the following symptoms: fever, arthralgia, leuco- and thrombopenia, liverfunction abnormalities (SGOT, SGPT), and rise in serum creatinine. One patient had a biopsy proved CMV pneumonia. CMV isolations (in urine) were performed in 8 out of the 9 patients with an active CMV infection. In 5 of these 8 patients CMV could be isolated.

Complement studies

Serum C3, C4 and FB were measured by rate nephelometry (Beckmann ICS Analyzer II, Fullerton, Ca, USA). In our laboratory the normal values are 46-160 mg/dl, 10-35 mg/dl and 18-46 mg/dl respectively. To block the C-activation *in vitro* for the determination of C3d, blood was collected in iced tubes. While the samples were kept on ice, the blood was allowed to clot for two hours before centrifugation in a cooled centrifuge. The samples were subsequently stored at -80°C until the moment when the determinations were made.

C3d ($\propto$2D)measurements were made by electroimmunodiffusion as described elsewhere (12). Anti C3d antiserum was provided by the Red Cross Bloodtransfusion Service, Amsterdam, The Netherlands. Although in the literature $\propto$2D has been equated with C3d this identification could be not fully correct. Recently it has been questioned whether C3d is formed in vivo at all (16). By using monoclonal anti-C3 antibodies Lachmann et al. suggested that in vivo only C3d,g is formed (16). However, for the sake of convenience, we will designate the activation product in the results obtained as C3d. Our standard C3d was kindly quantitated by M.R. Daha (University of Leiden, The Netherlands), and appeared to be 0.90 μg/ml. In our laboratory the normal values for C3d are 0.72-4.68 μg/ml. The intra and extraplate variation coefficient were less than 5%. Delta C3d was defined as the percentage increment of C3d at the time of R or CMV infection as compared with the level in the week before R or CMV infection.

Statistical analysis

For differences between groups of patients or between patients and controls, Wilcoxon's rank sum test was used.

RESULTS

Serum complement levels C3, C4 and FB

At the time of rejection the mean C3 level was 116 mg/dl (range 72-164 mg/dl), the mean C4 level 22.9 mg/dl (range 15.7-34 mg/dl) and the mean FB level was 28 mg/dl (range 21-45 mg/dl). During CMV infection the mean C3 level was 125 mg/dl (range 64.5-185 mg/dl), the mean C4 level 22.6 mg/dl (range 8.1-44.1 mg/dl) and the mean FB level was 32 mg/dl (range 13.4-49.7 mg/dl). There were no differences between the levels of the individual C-factors during R or CMV infection.

C3d levels

During the first 2-3 days after transplantation C3d levels decreased in all patients, thereafter C3d levels stabilized (fig. 1). During CMV infection C3d

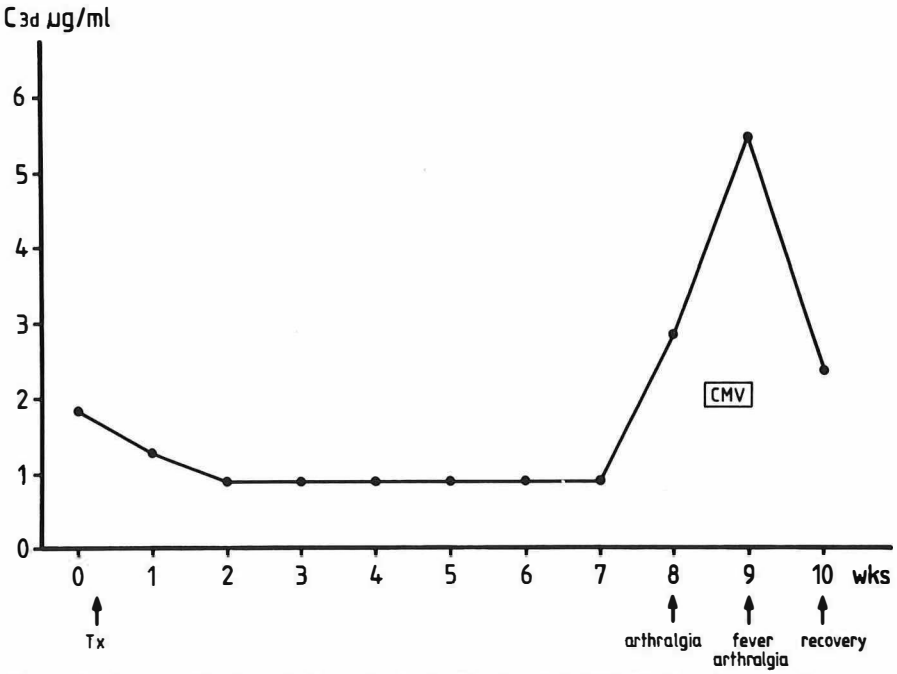
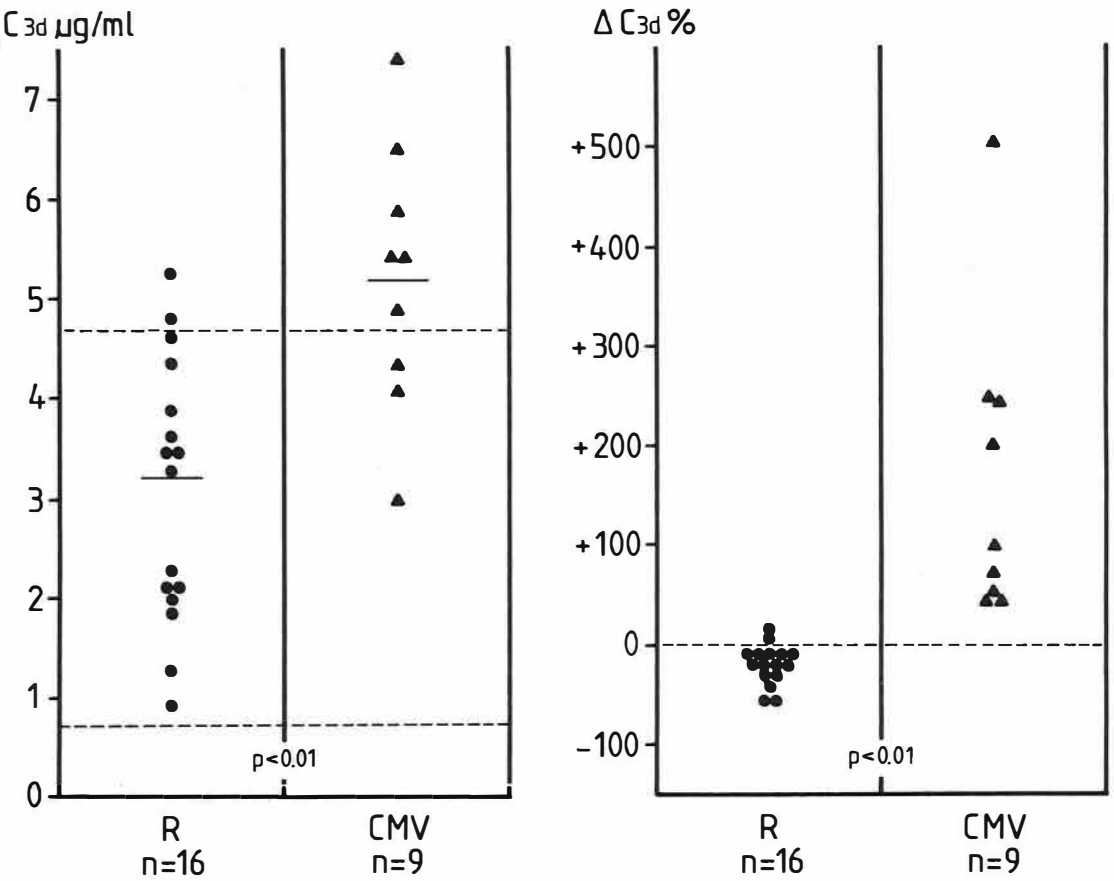


Figure 1. Course of C3d level of a patient who developed CMV infection 8 weeks after transplantation.

levels rose rapidly and significantly (fig. 1 and 2). On the contrary there were no such positive changes during rejection (fig. 2). Moreover there were significantly

Figure 2.

C3d levels during R and CMV infection, △C3d during R and CMV infection.



higher C3d levels during CMV infection in comparison with rejection (fig. 2). The changes in C3d levels ($\triangle$C3d) when R or a CMV infection occurred are also given in fig. 2. Delta C3d was significantly higher during CMV infection as compared to R. Nine patients showed only R without CMV infection (patient nos. 1,2,3, 8,9,10,11,17 and 18). Two patients (nos. 19 and 20) had neither R nor CMV infection. The highest level of C3d of patient no. 19 was 2.52 μg/ml and of patient no. 20, 3.98 μg/ml. Six patients suffered from an acute allograft rejection with a subsequent CMV infection (13-64 days after R; patient nos. 4,5,6,13,15 and 16). The differences between $\triangle$C3d at the time of R and $\triangle$C3d at the time of the CMV infection in these 6 patients are given in fig. 3. There were no differences in $\triangle$C3d in the prednisolone/azathioprine group as compared to the cyclosporin-treated group or differences in $\triangle$C3d between those with primary CMV infections and the group of patients with reactivation of CMV. Finally, no correlation whatsoever was observed between the level of IgG-antibodies against CMV-EA and LA, or the severity of the CMV infection and the level of C3d.

DISCUSSION

Our data are consistent with complement (C) activation during an active CMV infection in renal transplant recipients. In contrast no activation of C could be demonstrated during R. Two pathways of C activation have been defined, the classical and the alternative, or properdin, pathway; they share the central C-factor C3, after the activation of which the final common pathway leads to membrane damage (3,17).

A number of investigators have studied the role of C in the rejection process of renal transplant recipients, and the diagnostic value of C determinations during rejection episodes. Yokoyama et al. found depression of C3 and C4 levels as well as depression of the whole hemolytic activity of C (CH50), and to a lesser degree a depression in C1 and C2 levels (18). Carpenter et al. found biphasic elevation followed by depression of C3 (19). In all cases the serum complement levels were not depressed prior to the clinical rejection episode and often did not decline until a week or more after rejection. It is possible that this decline in serum complement levels was the result of therapy that was initiated after R was diagnosed (20), since steroids can inhibit the C-system (21). Shehadeh et al. have emphasized the instability of the serum levels of C3 and C4 that accompanies R episodes (22). From all published data Carpenter concluded that, from a diagnostic point of view, serum complement determinations are of little value in the early diagnosis of a pending rejection episode (23).

There are many reports on the role of antibody mediated destruction of virus-infected cells and the role of C in this process as reviewed by Sissons et al. (24).

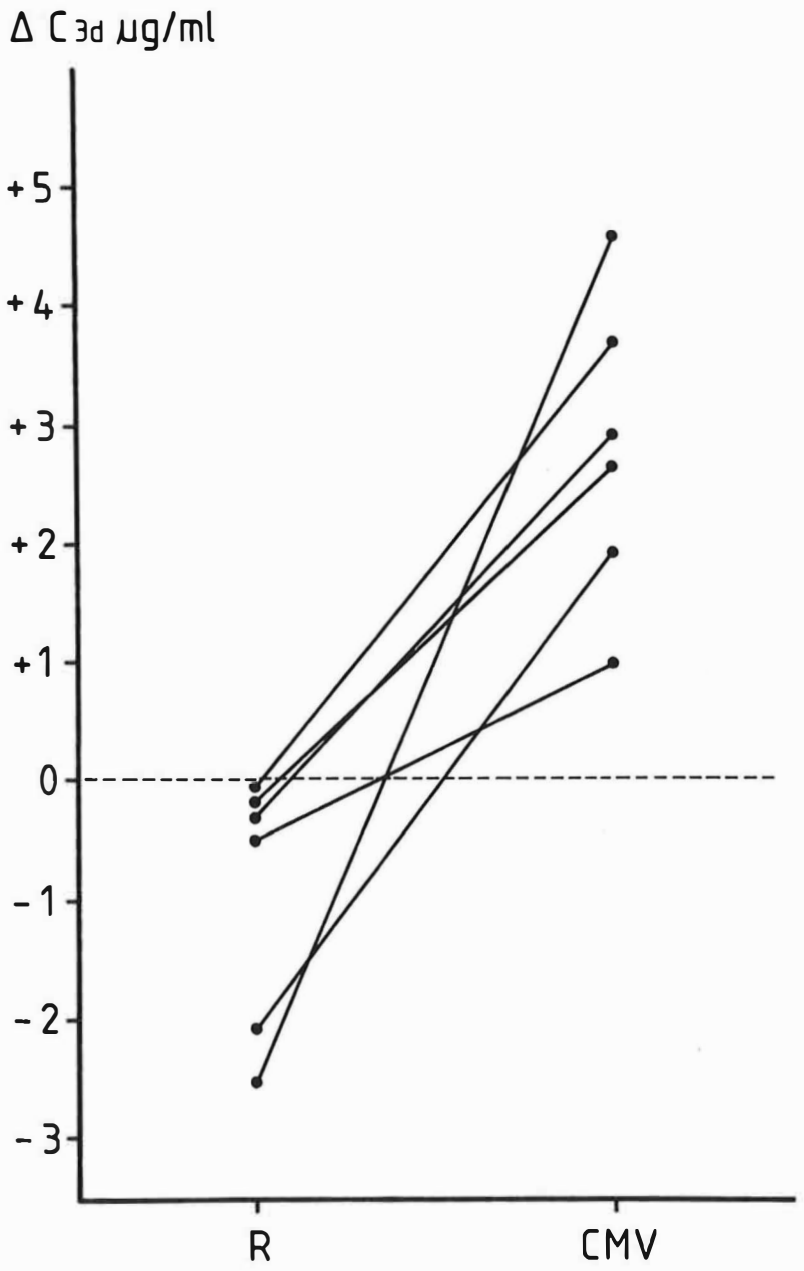


Figure 3. Course of ΔC3d of the 6 patients who suffered from R and subsequently of CMV infection (ΔC3d µg/ml).

Although most evidence indicates that the classical pathway of C is important for inactivation of viruses (4), virus-infected cells can activate the alternative C-path way independently of IgG, but antibody is required for subsequent lysis of the cells (24). Chatterjee and co-workers studied the C-system during CMV infection in renal transplant recipients (9). They found a depression of the serum level of FB together with FB split products in 5 out of 7 patients with a CMV infection (9). During R they could demonstrate FB split products in only one patient. Because depression of FB could also be caused by impaired synthesis (e.g liver dysfunction (9)) or by the corticosteroid treatment (20), it is not clear whether this depression reflects activation of the C-system. The finding of FB split products (not quantitatively measured) suggests activation of the alternative route of the C-system during a CMV infection (9). On the other hand, IgM immune complex-like material can be found in the circulation of renal transplant recipients during a CMV infection (8) which could activate the classical pathway of C.

In our patients the levels of C3, C4 and FB showed great instability during R or CMV infection without any consistent pattern. We could not demonstrate a depression of the serum level of FB during CMV found by Chatterjee et al. (9). Because, as mentioned before, depression of an individual C factor can be caused by something different from activation of the C-system it seems more adequate to look for C-activation products, for instance C3d (10-14). Elevation of the level of C3d can reflect activation of the classical as well as the alternative pathway. In our study all patients with a CMV infection showed a marked increase in the level of C3d, while during R the levels of C3d either remained stable or showed a small decrease. A few days after the onset of R, the C3d levels even became more depressed, which may be related to the anti-rejection treatment that was started (21). Interestingly, two patients (nos. 7 and 16) were initially misdiagnosed as having R because of the clinical signs, after which an anti-rejection treatment was given. Despite the augmentation of the oral prednisolone dosage the C3d levels in these patients showed a sharp increase. Later it became evident that both patients suffered from an active CMV infection (rise in titers of antibodies against CMV-EA and -LA, and clinical signs of CMV infection). Furthermore a typical CMV glomerulopathy (2) was demonstrated in the biopsy of the graft of patient no. 16. Three of the 9 patients with an active CMV infection had a normal C3d level during the infection, but using the patient as the control, the C3d level rose markedly during the CMV infection as compared with the level in the week before the CMV infection (fig. 2). Six patients had R and subsequently an active CMV infection 13-64 days after R. From fig. 3 it is clear that in all these patients the C-system acted differently during R and during the CMV infection.

We conclude that serial measurements of C3d help to differentiate between R and CMV infection after renal transplantation.

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CHAPTER 4

COMPLEMENT ACTIVATION DURING AN ACTIVE CYTOMEGALOVIRUS INFECTION AFTER RENAL TRANSPLANTATION: DUE TO CIRCULATING IMMUNE COMPLEXES OR ALTERNATIVE PATHWAY ACTIVATION?

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COMPLEMENT ACTIVATION DURING AN ACTIVE CYTOMEGALOVIRUS INFECTION AFTER RENAL TRANSPLANTATION: DUE TO CIRCULATING IMMUNE COMPLEXES OR ALTERNATIVE PATHWAY ACTIVATION?

Willem J. van Son*, Wim van der Bij**, Adam M. Tegzess*, Jetske Anema**, Marijke van der Giessen**, Gjalt K. van der Hem***, and T. Hauw The**.

From the Renal Transplantation Unit* and the Departments of Clinical Immunology** and Nephrology***, University Hospital Groningen, The Netherlands.

SUMMARY

In 32 patients with a renal allograft, serial determinations after transplantation were made of C3d, the stable conversion product of the complement factor C3, as well as serial measurements of the anaphylatoxin C3a des arg. Furthermore serial determinations were made on the presence of circulating immune complexes using 3 different assays (C1q binding assay, PEG precipitation test, and indirect granulocyte phagocytosis test). Twenty patients were studied during an active cytomegalovirus (CMV) infection, and 12 patients were studied during allograft rejection or during stable phase after renal transplantation. In 12 patients with a CMV infection serial measurements were made of AP50 (alternative pathway of complement). During an active CMV infection elevated C3d as well as elevated C3a des arg levels were found and not in the control group ($p < 0.01$). In 8 out of the 12 patients tested, with a CMV infection, activation of the alternative pathway could be demonstrated together with the elevated levels of C3d and C3a des arg. Circulating immune complexes were found to be positive in 15 out of the 20 patients with a CMV infection (both primary and secondary infections), and in 2 out of 12 patients of the control group. The complement activation found in the CMV group was *not* related to the presence of circulating immune complex-like material, since complement activation was present in advance of the appearance of the immune complexes, suggesting that complement activation was not due to classical pathway activation by those complexes.

We conclude that our data are consistent with complement activation and the

formation of biologically active peptides like C3a des arg in patients with an active CMV infection, and most likely are caused by activation of the alternative pathway of complement. The presence of circulating immune complexes was rather unspecific and not related to complement activation.

INTRODUCTION

An active cytomegalovirus (CMV) infection is frequently seen after renal transplantation, and is stated to have a negative influence on graft survival (1-3). While this high incidence seems to be modified by the introduction of the immunosuppressive agent cyclosporine A (CsA) (4), CMV infection after renal transplantation still is an important factor influencing graft survival (5,6). Results from experimental and clinical studies suggest that the integrity of the complement (C) system is important for the defence against micro-organisms and viruses (7-11). There are many reports on the role of antibody-mediated destruction of virus-infected cells and the role of C in this process, as reviewed by Sissons et al. (12). It is suggested that both pathways of C-activation might be implicated in the process of neutralization and lysis of viruses and virus-infected cells (11).

Complement is activated during an active cytomegalovirus infection after renal transplantation and, as we reported earlier (13), not during allograft rejection. From this study it was not possible to elucidate whether the classical or the alternative pathway of C was implicated in the C-activation in these patients (13).

Circulating immune complexes have been demonstrated in sera of patients with a congenital or natal CMV infection (14), and recently immune complex-like material could be demonstrated in the circulation of patients with a primary CMV infection after renal transplantation (15). In order to investigate whether the classical pathway of C (for instance via circulating immune complexes) or the alternative pathway of C is involved in the C-activation during a CMV infection, we performed serial measurements of complement breakdown products, as well as studies on the alternative pathway of C together with serial studies on the presence of circulating immune complexes in patients with an active CMV infection after renal transplantation.

MATERIALS AND METHODS

Thirty-two patients were studied (18 men and 14 women; age 18-62 years, mean 31). The studies were started immediately after transplantation and ended four months post-transplant. Thirty-one patients received a cadaveric graft, while 1

patient received a kidney from his one haplo-type mismatched mother. The immunosuppressive regimen consisted of cyclosporine A with (second transplants and patients with more than 60% cytotoxic antibodies) or without (first transplants) prednisolone. The patient that recieved his kidney from his mother was treated with prednisolone and azathioprine. Twenty patients had an active CMV infection in the postoperative period, 12 patients (controls) were studied either during a stable phase (n = 4) or during allograft rejection (n = 8). Ten patients had a primary infection, while 10 patients had a reactivation of the virus (secondary infection). An active CMV infection was defined as a fourfold or greater rise in antibodies to CMV early antigens (EA) and late antigens (LA). Antibodies (IgG and IgM) against EA and LA were detected by enzyme-linked immunosorbent assay as described elsewhere (16). Blood was drawn for detection of antibodies to CMV EA and LA before transplantation and once a week thereafter in all patients. The patients were considered to be seronegative for CMV when the titers of antibodies to CMV EA and LA were less than 2% of a standard serum. Three patients were asymptomatic during the CMV infection. All symptomatic patients with a serologically active CMV infection had one or more of the following symptoms: fever, arthralgia, leukopenia, thrombopenia, liverfunction abnormalities (SGOT and SGPT) or a rise in serum creatinine. One patient had pneumatosis intestinalis and bleeding ulcers of the esophagus, possibly related to the CMV infection (17). CMV was cultured from these ulcers. CMV isolation (blood, urine) was performed once a week in all patients. In 12 patients CMV could be isolated during an active CMV infection (table I). Allograft rejection was defined as a rapid increase in serum creatinine of 20% or more together with one or more of the following: a swollen painful kidney; fever; decrease in urinary output; salt retention; and findings compatible with rejection in a renal biopsy in the absence of post-renal-transplant abnormalities or signs of CsA toxicity. Anti-rejection treatment consisted of a bolus of 1000 mg methylprednisolon given i.v. on three successive days.

Complement studies

Complement breakdown product of C3: C3d

Blood was drawn for serial measurements of complement C3d in all patients once a week after renal transplantation. In order to study whether C is activated *in vivo* we performed serial determinations of C3d, a stable breakdown product of C3 (18-20). To block the C-activation *in vitro* for the determination of C3d, blood was collected in iced tubes. While the samples were kept on ice, the blood was

allowed to clot for 2 hours before centrifugation in a cooled centrifuge. The samples were subsequently stored at –80°C until the moment when the determinations were made. C3d (∝2D) measurements were made by electro-immunodiffusion, as described elsewhere (20). Anti-C3d antiserum was provided by the Red Cross Bloodtransfusion Service, Amsterdam, The Netherlands. Although in the literature ∝2D has been equated with C3d, this identification might be not fully correct. Recently it has been questioned whether C3d is formed in vivo at all (21). By using monoclonal anti C3d antibodies Lachmann et al. suggested that in vivo only C3d,g is formed (21). However, for the sake of convenience, we will designate the activation product in the results obtained as C3d. In our laboratory the normal values for C3d are 0.72-4.68 μg/ml. The intra- and inter-test variation coefficients were less than 5%. Delta C3d (△C3d) was defined as the actual increase in C3d levels found during an active CMV infection (as compared to the levels found *before* the infection).

Measurements of the biologically active peptide of C-activation: the anaphylatoxin C3a (measured as C3a des arg)

Serial measurements were made of C3a des arg, the biologically active fragment that is formed during C-activation (22). Blood was drawn once a week after transplantation in iced tubes containing 10 mM Na_2 EDTA to block the activation of C *in vitro*. After centrifugation the samples were stored (within 30 minutes of venipuncture) at –80°C until the determinations were made. C3a des arg was measured by radioimmunoassay with ^{125}I C3a des arg (23) using the Upjohn Kit (Upjohn Diagnostics, Kalamazoo MI 49001, USA). Delta C3a des arg was defined as the actual increase in C3a des arg levels found during an active CMV infection (as compared to the levels found before the infection). We designated the maximum level of C3a des arg that could be obtained with this test as 1000 ng/ml because further dilution of the samples may give unpredictable results (24).

Studies on the hemolytic activity of the alternative pathway of C-activation: AP50

We were able to perform serial measurements of the alternative pathway activation in 12 out of the 20 patients with an active CMV infection. These measurements were kindly performed by Dr. M.R. Daha, State University Leiden, The Netherlands, using an assay as previously described (25). The normal values in this laboratory for AP50 are 8-24 U/ml.

Soluble immune complexes (CIC)

Serial measurements on the presence of CIC were performed in all patients. In our laboratory we use three different methods for the detection of CIC:

a) *a granulocyte phagocytosis test* (IGPT) as an indirect procedure for the detection of CIC: results are expressed as number of cells containing 6 or more fluorescent granules per 1000 cells (IgG and IgM). Normal values: <40.
b) a sensitive *solid phase Clq binding assay* which uses an enzyme-substrate reaction for the development: results are expressed as μg eq of aggregates of human IgG. Normal values: 19 μg eq $\pm$ 5.
c) a simple *PEG precipitation test:* the quantity of precipitate is measured by optical density at 280 nm. Normal values 70-270.

The exact procedure of all three techniques has been recently described by two of us (26).

Statistical analysis

Statistical analysis was performed using Wilcoxon's Rank sum test.

RESULTS

Twenty patients suffered from an active CMV infection in the post-operative period. Ten patients had a primary infection while ten patients had a secondary infection with CMV. Only 3 patients were asymptomatic during the CMV infection. Clinical details, as well as the results of the C3d, C3a des arg, AP50 and CIC measurements are given in table I.

The results of the C3d determination are given in figure 1. During CMV infection C3d ranged from 2.7 to 20 μg/ml, with a mean value of 9.19 μg/ml and a median of 7.5 μg/ml. In contrast, all patients of the control group had C3d levels in the normal range (range 1.0-3.9 μg/ml; mean value 2.5 μg/ml; median 2.35 μg/ml). The difference between the 2 groups was statistically significant ($p < 0.01$). The $\triangle$ C3d found during CMV infection ranged from 1.7-11.8 with a mean value of 6.28 μg/ml. No statistical differences could be demonstrated in C3d or $\triangle$ C3d between patients with a primary infection and those with a reactivation of the virus.

The results of the anaphylatoxin C3a des arg determinations are given in figure 2. The C3a des arg levels of the patients with a CMV infection ranged from 228 to 1000 ng/ml (mean 630; median 513 ng/ml). In the control group C3a des arg

Table I. Clinical details, C3d, C3a des arg levels, circulating immune complexes of the CMV group (maximum levels), and AP50 (nadir values).

Pat. no.	Age (yrs)	Sex	Type of infection	S/A	CMV isolation	C3d µg/ml	C3a des arg (ng/ml)	AP50 U/ml	IGPT-score IgG	IgM	C1q-ELISA	PEG OD 280 nm	Clinical outcome
1	60	F	primary	S	pos(U.B.ulcer)	3.7	345	7	320	540	neg	140	complete recovery
2	62	F	primary	A	pos(B)	5.5	318	25	400	400	neg	220	complete recovery
3	54	F	primary	A	pos(U.B)	12.8	560	5	40	200	neg	240	complete recovery
4	21	M	primary	S	neg	17.1	448	16	40	80	neg	292	complete recovery
5	26	M	secondary	A	pos(U.B)	5.7	210	1	30	neg	neg	145	complete recovery
6	52	M	secondary	S	neg	7.7	>1000	3	50	100	neg	324	complete recovery
7	27	M	secondary	S	pos(B)	16.1	477	1	1000	330	neg	242	complete recovery
8	33	M	secondary	S	neg	3.9	450	ND	190	230	neg	249	complete recovery
9	56	M	secondary	S	neg	2.7	228	37	330	230	neg	671	complete recovery; chronic rejection
10	38	F	secondary	S	neg	20	>1000	9	10	neg	neg	231	complete recovery
11	23	M	primary	S	pos(U)	5.0	>1000	5	20	5	neg	270	complete recovery; chronic rejection
12	33	F	primary	S	neg	7.3	>1000	31	830	110	neg	565	complete recovery
13	53	F	primary	S	pos(lung,liver)	8.3	>1000	5	10	10	neg	262	died: sepsis, asperg.
14	27	F	secondary	S	pos(U.B)	14.7	>1000	ND	660	580	neg	255	complete recovery
15	54	M	secondary	S	pos(U.B)	15.3	>1000	ND	40	50	neg	ND*	died: sepsis (E.coli, candida)
16	44	M	primary	S	pos(U.B)	6.6	440	ND	60	250	neg	827	complete recovery
17	29	M	secondary	S	neg	8.2	204	ND	20	30	neg	679	complete revovery
18	31	M	primary	S	pos(B)	6.2	550	ND	30	10	neg	550	complete recovery
19	48	F	secondary	S	neg	4.5	368	ND	40	50	neg	202	complete recovery
20	23	M	primary	S	pos(B)	12.0	>1000	ND	310	720	neg	727	complete recovery

ND = not done; ND* = not done (lipemic serum); F = female; M = male; S = symptomatic; A = asymptomatic; U = urine; B = blood

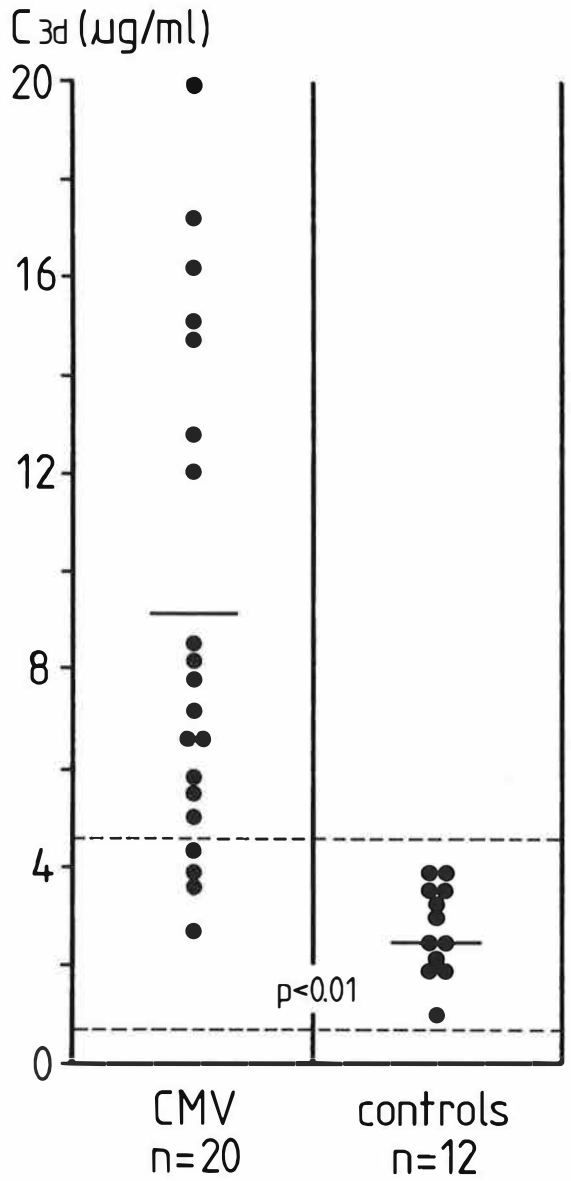

Figure 1. Shown are C3d levels of the patients with an active cytomegalovirus infection and of the control group. Highest and mean values of C3d are given for both groups. Range of normal values are in the area between the dotted lines.

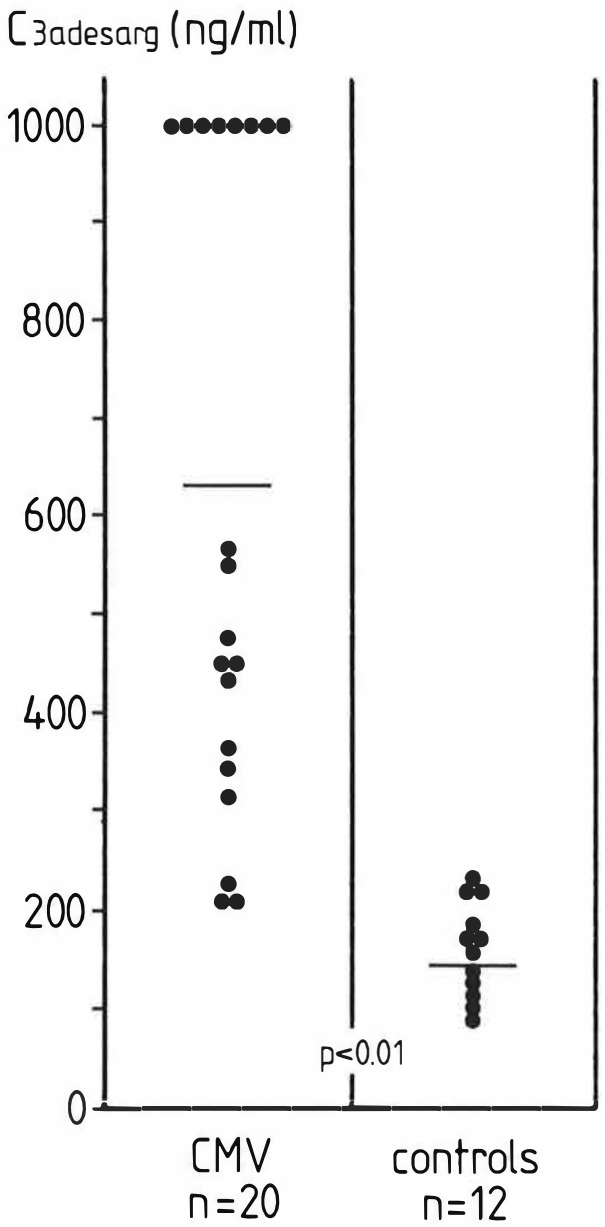

Figure 2. Shown are C3a des arg levels of the patients with an active cytomegalovirus infection and of the control group. Highest and mean values of C3a des arg are given for both groups.

ranged from 98 to 225 ng/ml (mean, 144; median 132 ng/ml). The difference between the 2 groups was statistically significant ($p < 0.01$). The Δ C3a found during CMV infection ranged from 77 to 847 ng/ml with a mean value of 426 ng/ml

No statistically significant differences could be demonstrated between patients with primary CMV infections and those who had a reactivation of the virus.

In 12 patients AP50 was studied (see table I), and was found to be abnormally low in 8 out of the 12 patients. AP50 ranged from 1 to 37 U/ml. One out of the 4 patients with normal AP50 values also did not have elevated C3d and C3a des arg levels. Of the 2 patients that died after the active CMV infection, complement levels before the final septic episode are given in table I.

Circulating immune complex-like material (CIC)

In 15 out of the 20 patients studied circulating immune complex-like material could be demonstrated with one or more assays during CMV infection (see table I). In 10 patients only one test was found to be positive, while in 5 patients two of the CIC assays were positive. Since the C1q binding assay was negative in all patients tested, none of the patients were found to be positive in all 3 assays used in this study (see table II). Fourteen out of the 20 patients with a CMV infection had a positive IgM-IGPT, while 9 patients had a positive IgG-IGPT. Ten patients had a positive IgG- as well as a positive IgM-IGPT-test. The PEG precipitation test was positive in 9 out of the 20 patients with a CMV infection.

Table II. Number of positive CIC tests in patients with a CMV infection and of the control group.

no tests positive	0	1	2	3
CMV group (n = 20)	5	10	5	0
Controls (n = 12)	10	2	0	0

In contrast only 2 patients of the control group had a positive CIC test (see table II). Both patients had only a positive IGPT test (one patient had both IgM- as well as a positive IgG-IGPT). The other patient had only a positive IgM-IGPT). In both patients the positive test correlated well with a period of allograft rejection. An example of the course of the CIC tests as well as the course of the C3a des arg and C3d levels in a patient with a CMV infection is given in figure 3. An example of the course of C3a des arg levels, AP50 levels together with the course of CIC is given in figure 4.

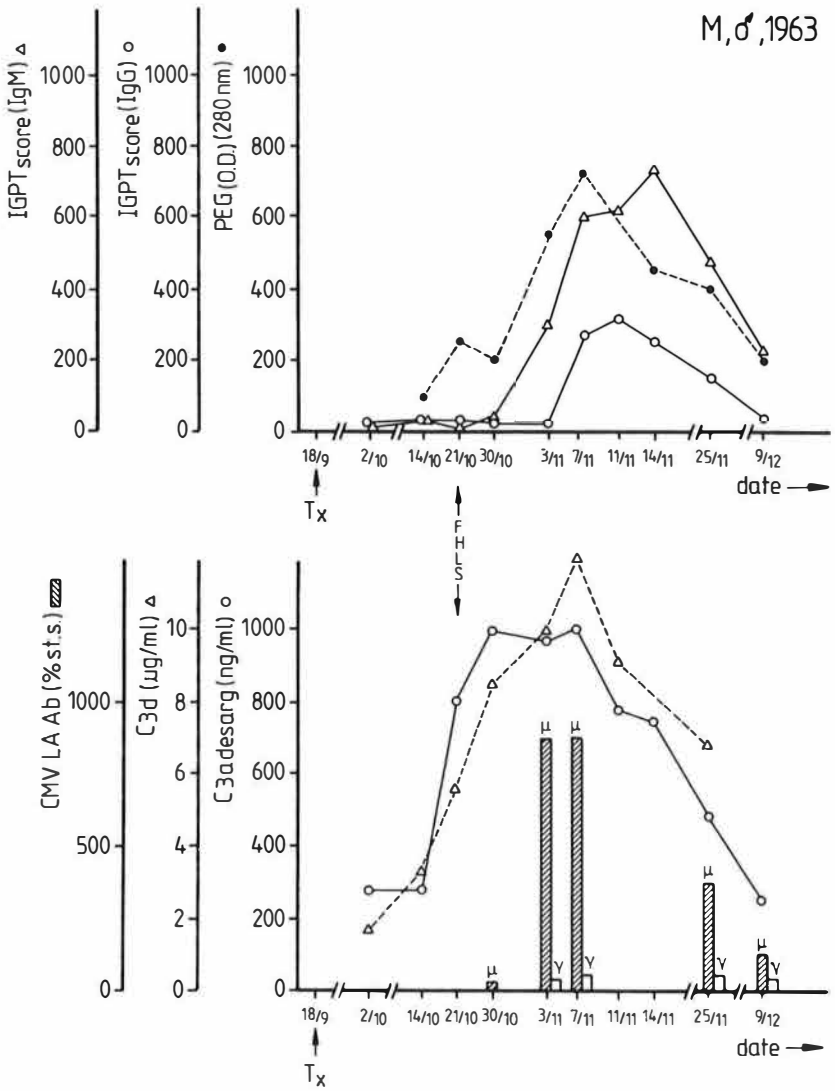

Figure 3. Course of levels of circulating immune complexes (upper half), levels of C3d as well as C3a des arg (lower half) in a patient with a cytomegalovirus infection.
F = fever, H = hematologic abnormalities (leucopenia, thrombopenia), L = liver function abnormalities, S = moment when cytomegalovirus could be isolated from the blood followed one week later by seroconversion. μ: IgM antibodies, γ: IgG antibodies against CMV.

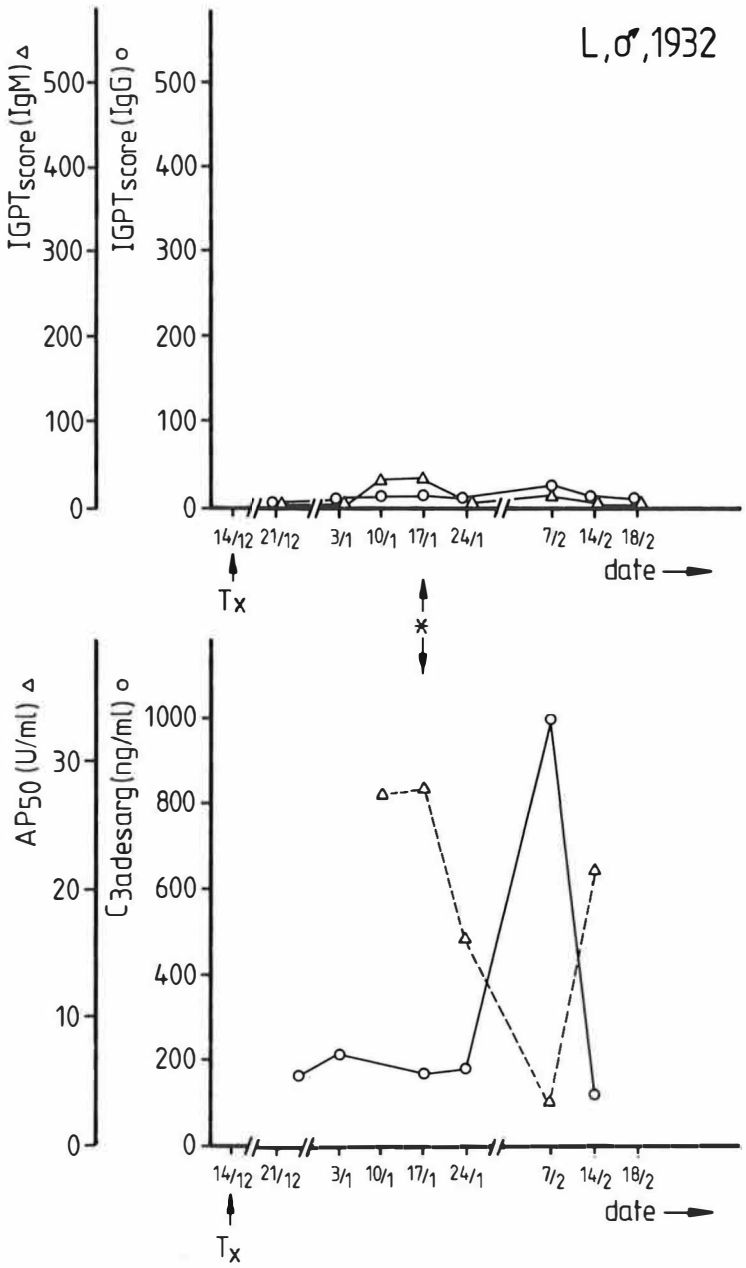

Figure 4. Course of levels of circulating immune complexes (upper half), levels of C3a des arg as well as AP50 levels (lower half) in a patient with a cytomegalovirus infection.
*: moment of seroconversion.

DISCUSSION

Our data are consistent with complement activation and formation of biologically active peptides like C3a des arg during CMV infection after renal transplantation. Moreover, during CMV infection we found evidence for immune complex-like material (both IgG and IgM) in the circulation of these patients, while only 2 out of the 12 patients of the control population had evidence for such complexes throughout the study period. None of the patients of the control group had more than one positive assay for CIC, while in the CMV group 5 patients had more than one assay positive for CIC (see table II).

There are several types of natural immunity to viruses and virus-specific infected cells. In some instances monocytes, macrophages and polymorphonuclear cells directly recognize virus infected cells and perhaps viruses in the absence of antibody and complement (11). As to the relevance of humoral immunity to viruses, many investigations stress the importance of antibody and complement mediated neutralization and lysis of virus and virus-infected cells (reviewed by Sissons, 12). Viral and virus related cellular surface structures are antigenic and elicit both humoral and cellular immune responses (11). Virus particles coated with IgG or IGM antibody may behave like typical immune complexes and activate the classical pathway of complement (11). Virus-antibody complexes may also occur as a consequence of antibody induced redistribution ('capping') of viral antigens on the surface of virus infected cells, which are subsequently shed into the circulation (27-30). In addition to immune activation of the classical pathway of C some viruses are capable of activating the classical pathway in the absence of antibody (11). For instance, retroviruses bind directly to C1q thereby activating the classical pathway of complement in the absence of antibody (31,32). In mammals and man, the alternative pathway of complement is activated by Simian virus 5 (33), influenza virus (33,34), Sinbis (33), vesicular stomatitis (33) and Epstein-Barr viruses (35). Alternative pathway activation by several of the viruses including influenza requires antibody, while Sinbis and Epstein-Barr viruses are capable of activating the alternative pathway of complement in absorbed and/or agammaglobulinemic sera (11,35). Some viruses are able to activate both pathways of complement for instance Sinbis Virus (36). Apart from the role of complement activation in neutralizing and lysis of virus and virus infected cells, the activated complement system also has the ability to induce an acute inflammatory response, since the anaphylatoxins like C3a des arg formed during complement activation can alter vessel permeability and produce edema, induce smooth muscle contraction, stimulate influx of leucocytes and facilitate phagocytosis and the release of secondary mediators (22,37). Furthermore, complement may be important in the clearance process of the immune complexes formed during infection (38).

In contrast to the above mentioned favourable effects of complement activation, its activation may also have detrimental effects and may induce immune complex disease (39). It is possible that the formation of anaphylactoid factors like C3a des arg, found during CMV infection, might be important in the pathophysiology of some of the protean manifestations of the CMV-syndrome. Complement activation during CMV infection could, for instance, be responsible for the frequently observed pulmonary dysfunction during an active CMV infection after renal transplantation (40), since anaphylatoxins like C3a des arg and C5a des arg are known to play an important role in a diversity of pulmonary syndromes (41,42).

In our present study all but one (patient no. 9, see table I) showed evidence for complement activation during CMV infection. In contrast none of the patients of the control population showed evidence for complement involvement. Most of the patients of the CMV group that showed C-activation had evidence for alternative pathway activation (see table I and fig. 4). The results shown in figure 4 clearly demonstrate alternative pathway activation without the presence of circulating immune complexes. In our earlier work we demonstrated normal C4 levels during C-activation in patients with an active CMV infection (13) which is also in favour of alternative pathway activation. Seventy-five percent of the patients of the CMV group showed evidence of circulating immune complex-like material. In contrast to the results of Baldwin et al. (15) who showed evidence of IgM immune complex-like material only in patients with a primary cytomegalovirus infection we found evidence for both IgG and IgM immune complex-like material in patients with a primary infection, but also in patients with a reactivation of the virus. Interestingly, one assay (the C1q binding assay) was found to be negative in all patients. The IGPT test primarily detects, and is highly sensitive for immune complexes formed in antibody excess, while in contrast the C1q-ELISA detects immune complexes in slight antigen excess (26). The negative results with the C1q-ELISA test could for instance have been caused by the possible antibody excess in our patients with a CMV infection. All of the patients with a CMV infection who had negative results in all three CIC assays had evidence of C-activation while in those patients who did have immune complex-like material in their circulation complement activation occurred *in advance* of the appearance of immune complex-like material in the circulation (example: fig. 3). The discrepancy in time between the moment of C-activation and the appearance of CIC could indicate that the complement activation, observed in the patients with a CMV infection, was *not* due to classical pathway activation via circulating immune complexes. Futhermore, the 2 patients of the control group that had a positive IGPT test during allograft rejection showed no evidence of C-activation at all.

In conclusion, complement is activated during an active CMV infection after

renal transplantation. During infection biologically active peptides like the anaphylactoid factor C3a des arg are also demonstrable in the circulation. Furthermore, immune complex-like material is found in the circulation of patients with a primary infection as well as in patients with a secondary infection, but also in some of the patients with allograft rejection. Since we found no correlation between the moment of C-activation and the appearance of the CIC like material, and most of the patients tested for alternative pathway involvement, had evidence for alternative pathway activation, we postulate that the C-activation observed during an active CMV infection after renal transplantation most likely is caused by alternative pathway activation. Since not all of the patients showed alternative pathway involvement, the possible additional role of direct classical pathway activation remains to be clarified.

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CHAPTER 5

CYTOMEGALOVIRUS INFECTION AFTER RENAL TRANSPLANTATION: PULMONARY DYSFUNCTION MEASURED BY DECREASED DIFFUSING CAPACITY FOR CARBON MONOXIDE (KCO) IN PATIENS WITH SYMPTOMATIC AND ASYMPTOMATIC INFECTION

CHAPTER 5

CYTOMEGALOVIRUS INFECTION AFTER RENAL TRANSPLANTATION: PULMONARY DYSFUNCTION MEASURED BY DECREASED DIFFUSING CAPACITY FOR CARBON MONOXIDE (KCO) IN PATIENS WITH SYMPTOMATIC AND ASYMPTOMATIC INFECTION.

Willem J. van Son*, Rafael Peset, Josée C. Duipmans, Thomas W. van der Mark, T. Hauw The** and Adam M. Tegzess*

From the Departments of Lung Function, Renal Transplantation* and Clinical Immunology** University Hospital Groningen, The Netherlands.

Cytomegalovirus (CMV) infection is frequently seen after cadaveric renal transplantation and its symptomatology may show a great variety (1,2). It may include fever, arthralgia, leucopenia and abnormalities in liver enzymes. To differentiate between an active CMV infection and acute allograft rejection can be very difficult because fever, a rise in serum creatinine, and a swollen, painful kidney as well as abnormalities in graft biopsies may be found in both conditions (1,3).

CMV pneumonitis can also be a potentially dangerous feature of the CMV syndrome posttransplant (2). The chest roentgenogram in patients with biopsy proven CMV pneumonitis may reveal a diffuse pattern, bronchopneumonia or, rarely, a lobar pattern (2). Measuring the carbon-monoxide transfer factor (TLCO) and Krogh's coefficient (KCO) are sensitive parameters for a range of diffuse interstitial pulmonary afflictions including Bleomycin induced pulmonary toxicity (4).

In this report we show in 8 patients with a cadaveric graft that KCO is decreased during an active CMV infection posttransplant, even without roentgenogram abnormalities and with normal arterial bloodgases. In a control group of 12 patients with a cadaveric graft without a CMV infection the KCO was significantly higher. The 20 patients (8 male, 12 female, age: 21-60 years, mean 37 years) were treated with cyclosporin A with or without prednisolone. The diagnosis active CMV infection was defined as a fourfold or greater rise in antibodies (IgG and IgM) against CMV early and late antigens by enzyme-linked-immunosorbent assay as described elsewhere (5). In 8 patients the diagnosis active

CMV infection was made (4 primary infections, 4 reactivations). Two patients were asymptomatic while 5 patients had only minor symptoms like fever and arthralgia during the CMV infection. One patient also had pneumatosis intestinalis and bleeding ulcers in the esophagus during the CMV infection. CMV was cultured from the ulcers in this patient. None of the 8 patients had pulmonary symptoms, roentgenographic abnormalities or abnormalities in the arterial bloodgases during the CMV infection.

In the control group (n = 12) without CMV, KCO was measured either during a stable phase after transplantation or during rejection. KCO was measured by a modified rebreathing technique as described by Clark et al. (6) using an anesthetic bag filled with a gas mixture with 10% Helium and 0.3% CO. All measurements were made with the patient in supine position after 15 minutes rest, and were carried out in triplicate. A correction was made for the hemoglobin concentration, designated as KCOc, by dividing KCO by the Hb constant (Table I). In both groups 5-19 measurement were made (mean 8). Statistical analyses were performed using the Student's-t-test. The results are given in table I. The differences in KCO between the two groups are significant with or without correction for the hemoglobin values. In figure 1 a typical course of the KCO in one patient with a CMV infection is given. When the patient became symptomatic (arthralgia, fever) the KCO dropped only to rise again when the patient's symptoms subsided. This patient had no pulmonary symptoms at all.

In summary, our data indicate that KCO is decreased during an active CMV infection posttransplant even without pulmonary symptoms, roentgenographic abnormalities, or arterial bloodgases. Our findings may indicate that every patient with an active CMV infection posttransplant has a subclinical pneumonitis possibly evolving to an overt pneumonitis. The patients with a large fall in KCO may prove to be a group of patients particularly at risk. Furthermore measuring of KCO by this simple bedside method could be of help in differentiating between an active CMV infection and rejection after renal transplantation. As to the pathogenesis of the drop in KCO during a CMV infection, one can only speculate. It could be a direct result of primary cytopathogenic effect of the CMV on the lung parenchyma. Another possibility could be that it reflects a complement mediated phenomenon as in Adult Respiratory Distress Syndrome (7), or in hemodialysis hypoxemia (8) because complement is also activated during an active CMV infection after renal transplantation (9).

Table I Mean KCO and KCOc values of patients with an active CMV infection after renal transplantation and of the control group.

	CMV (n = 8)	Controls (n = 12)	
mean age (yr)	38	36	N.S.
KCO mean mmol/min/KPa/l	1.02	1.45	p<0.005
SD	0.13	0.35	
KCOc mean $(\frac{KCOx14.6}{Hb})$ mmol/min/KPa/1	1.73	2.38	p<0.01
SD	0.52	0.54	
Hb mean (gr%)	9.3	9.3	N.S.
SD	1.29	1.26	

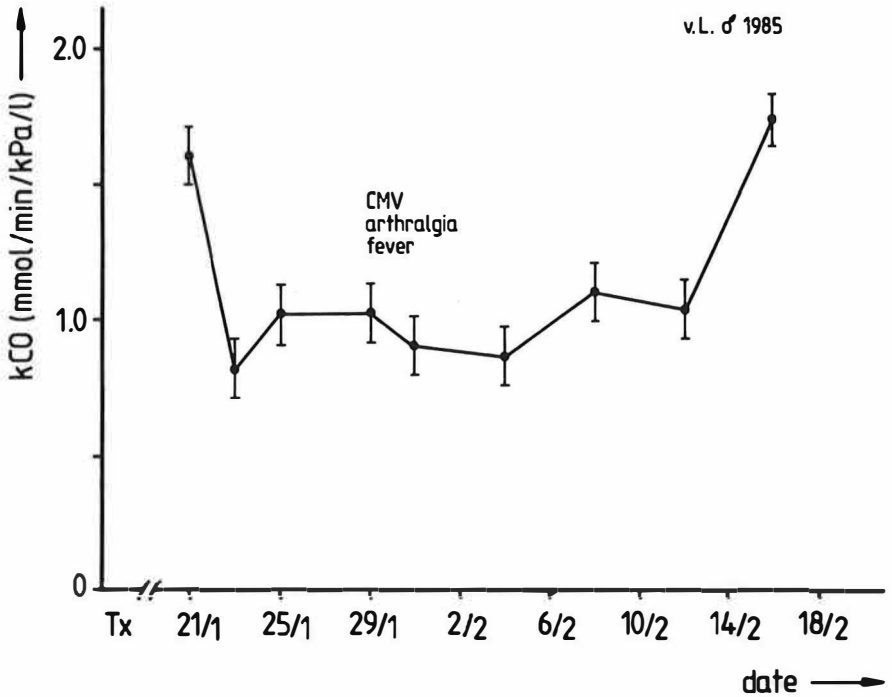

Figure 1. Course of KCO in a patient with a CMV infection. Mean values are given; bars represent confidence limits.

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CHAPTER 6

PULMONARY DYSFUNCTION IS COMMON DURING A CYTOMEGALOVIRUS INFECTION AFTER RENAL TRANSPLANTATION, EVEN IN ASYMPTOMATIC PATIENTS: POSSIBLE RELATIONSHIP WITH COMPLEMENT ACTIVATION

CHAPTER 6

PULMONARY DYSFUNCTION IS COMMON DURING A CYTOMEGALOVIRUS INFECTION AFTER RENAL TRANSPLANTATION, EVEN IN ASYMPTOMATIC PATIENTS: POSSIBLE RELATIONSHIP WITH COMPLEMENT ACTIVATION

Willem J. van Son*, Adam M. Tegzess*, T. Hauw The***, José Duipmans**, Maarten J.H. Slooff*, Thomas W. van der Mark** and Rafael Peset**

From the Departments of Renal Transplantation*, Lung Function** and Clinical Immunology***, University Hospital Groningen, The Netherlands.

SUMMARY

In 24 patients with a cadaveric renal allograft serial measurements after transplantation were made of the diffusing capacity for carbon monoxide (KCO) together with serial measurements of the stable conversion product of the complement factor C3, C3d, and determinations of the anaphylatoxin C3a. Twelve patients were studied during an active cytomegalovirus (CMV) infection, and 12 patients were studied during allograft rejection or during a stable phase after renal transplantation (control-subjects). No patient had pulmonary symptoms, abnormal chest radiographs or arterial blood gas determinations. During an active CMV infection KCO was significantly reduced as compared with the measurements made during allograft rejection or during a stable phase after renal transplantation. This was true both with ($p<0.01$) and without ($p<0.01$) correction for the hemoglobin concentration. Serum C3d levels were increased in 8 of the 12 patients with a CMV infection but not in any of the patients of the control population. In 8 patients with a CMV infection measurements were made of the anaphylatoxin C3a, and were found to be significantly higher than the levels in the control population ($p<0.01$).

We conclude that our data are consistent with pulmonary dysfunction in every patient with an active CMV infection. The concomitant findings of complement activation and formation of anaphylatoxins suggest a causal relationship of the complement activation and the decreased KCO, although further studies are

warranted to determine the exact role of complement in the pulmonary events during an active CMV infection after renal transplantation. Serial measurements of KCO with this simple bedside method can be of help in the sometimes difficult differential diagnosis between CMV infection and allograft rejection. Furthermore, the patients with the greatest drop in KCO might prove to be the patients who are at greatest risk to develop an overt CMV pneumonitis.

INTRODUCTION

Cytomegalovirus (CMV) infection is frequently seen after cadaveric renal transplantation, and its incidence has been stated as high as 43-92%, although most infections are asymptomatic (1,2). Although this high incidence seems to be modified by the introduction of the immunosuppressive agent cyclosporine A (CsA) (3), CMV infection after renal transplantation still is an important factor influencing the graft survival (4). The symptomatology of a CMV infection may show a great variety and may include fever, arthralgia, leucopenia, abnormalities in liver enzymes (1,2), and abnormalities in biopsies of the renal graft (5). CMV pneumonitis can also be a, potentially dangerous, feature of the CMV syndrome after transplantation (2). Although the exact pathogenesis of the CMV pneumonitis is not known (6), some investigators suggest that it could be an immunologically mediated phenomenon rather than the result of direct viral damage to the lung (7). During a CMV infection after renal transplantation the diffusing capacity of the lungs for carbon monoxide (KCO) tends to be decreased as we reported earlier (8).

In order to investigate whether this phenomenon is, in analogy with the pulmonary dysfunction during hemodialysis (9) and respiratory distress syndrome (10), complement (C) mediated, we studied the metabolism of the C system together with serial measurements of KCO in patients with a CMV infection after renal transplantation.

PATIENTS AND METHODS

Twenty-four patients were studied, 11 men and 13 women (age: 21-56 yrs, mean 39 yrs). All patients received a cadaveric graft. The immunosuppressive regimen consisted of cyclosporine A with (second transplants, or patients with cytotoxic antibodies) or without (first transplants) prednisolone. Twelve patients had an active CMV infection in the postoperative period, twelve patients were studied as control subjects either during a stable phase (n = 4) or during allograft rejection (n = 8). Five patients had a primary infection (seronegative before

transplantation), and 7 patients had a reactivation of the virus. None of them had pulmonary symptoms nor abnormal chest radiographs or arterial bloodgas determinations. None of the patients had signs of pulmonary congestion on physical examination, and none of them was taking medication during the study period that could alter the diffusing capacity of the lungs. Allograft rejection was defined as an increase in serum creatinine of 20% or more together with one or more of the following: a swollen, painful kidney; fever; decrease in urinary output; salt retention; and findings compatible with rejection in a renal biopsy in the absence of post-renal-transplant abnormalities. Antirejection treatment consisted of a bolus of 1000 mg methylprednisolone given i.v. three times on successive days. An active CMV infection was defined as a fourfold or greater rise in antibodies to CMV early antigens (EA) and late antigens (LA). Antibodies (IgG and IgM) against EA and LA were detected by enzyme-linked-immunosorbent assay as described elsewhere (11). Blood was drawn for detection of antibodies to CMV EA and LA before transplantation and once a week thereafter in all patients. The patients were considered to be seronegative for CMV when the titers of antibodies to CMV EA and LA were less than 1:40. Three patients were asymptomatic during CMV infection. All symptomatic patients with a serologically active CMV infection had one or more of the following symptoms: fever, arthralgia, leukopenia, thrombopenia, liver function abnormalities (SGOT and SGPT) or a rise in serum creatinine. One patient had pneumatosis intestinalis and bleeding ulcers of the esophagus; CMV was cultured from these ulcers. CMV isolation (blood, urine) was performed once a week in all patients. In 6 patients CMV could be isolated during an active CMV infection.

Diffusing capacity of the lungs for carbon monoxide (KCO)

The diffusing capacity of the lungs for carbon monoxide was measured by the modified rebreathing technique described by Clark and coworkers (12). A two way valve (13) was used for this purpose, connected to a 0.75 l anesthetic bag. The bag was filled with a gas mixture containing 0.3% CO and 10% Helium with the balance air, after flushing it three times with this gas mixture. The patient was asked to breath with a frequency of 30 per minute for 10 seconds. Thus 5 breathing manoeuvres were enough to achieve a good mixing in the bag. The measurements were carried out (in triplicate) with the patient in the supine position with the head at an angle of 30° with the horizontal after a rest period of 15 minutes in this position. The results were expressed as the Krogh's coefficient KCO, i.e. the diffusing capacity per liter lung volume. KCO was calculated with the use of the equation as described by Clark and coworkers (12). Because anemia was a frequent phenomenon in our transplant patients (this was both true for the patients with and without an active CMV infection), the results of KCO

Table I. Nadir KCO, nadir KCOc, △ KCO and △ KCOc of the CMV group and of the controls.

CMV (n = 12)	1	2	3	4	5	6	7	8	9	10	11	12	Mean ± SEM
nadir KCOc	1.56	1.54	1.04	1.20	1.52	0.83	1.44	1.48	1.09	0.81	1.37	1.90	1.32 ± 0.09
nadir KCO	0.92	1.00	0.92	0.94	1.01	0.59	0.75	0.83	0.51	0.49	0.79	1.16	0.83 ± 0.06
△ KCOc	0.79	1.11	0.84	0.87	0.72	1.44	1.01	1.49	2.05	2.28	0.84	0.61	1.17 ± 0.16
△ KCO	0.51	0.85	0.74	0.63	0.36	0.89	1.14	0.91	0.88	1.18	0.29	0.56	0.75 ± 0.08

Controls (n = 12)	1	2	3	4	5	6	7	8	9	10	11	12	Mean ± SEM
nadir KCOc	2.37	1.44	1.68	2.03	2.27	3.02	2.40	2.19	1.85	1.99	1.58	1.48	2.03 ± 0.13*
nadir KCO	1.38	0.88	1.14	1.17	1.26	1.79	1.38	1.29	1.33	1.62	1.23	0.84	1.28 ± 0.08*
△ KCOc	0.48	1.22	0.53	0.96	0.86	0.57	0.37	0.36	0.67	0.43	0.17	0.29	0.59 ± 0.08*
△ KCO	0.39	0.26	0.19	0.36	0.48	0.61	0.41	0.12	0.29	0.57	0.10	0.14	0.33 ± 0.05*

$$KCOc = KCO \times \frac{14.6}{Hb}$$

Mean age (yrs) CMV group 39; Controls 38: NS

Mean Hb (g/l) CMV group 93 (SD 1.29); controls 93 (SD 1.26): NS

*Significant difference ($p < 0.01$) in comparison with the CMV group

measurements have been corrected assuming a standard hemoglobin concentration of 14.6 g per 100 ml (designated as KCOc; table I). In both groups 5-19 measurements were made (mean 9). The KCO studies were started as soon as the patients left the isolation ward (at the moment when the ureteral stent as well as the bladder catheter had been removed; usually the 12th day after transplantation).

Measurements were carried out, if possible, at least once a week during the study period. When a CMV infection was suspected on clinical grounds measurements were carried out, whenever possible practically, more frequently. The KCO studies were continued in the outpatient clinic, and were stopped three months after transplantation when no CMV infection had occurred within this period or when the reconvalescence phase of a CMV infection was within this 3 months period. Measurements were continued after this 3 months period when a patient had a CMV infection at the end of the study period in order to follow the changes in KCO in the reconvalescence period of the infection. Three patients were excluded from the study; one patient with severe bronchial asthma and two patients with a lobar *bacterial* bronchopneumonia, all with overt chest radiographs abnormalities. Delta KCO and Δ KCOc were defined as the actual or percentual difference between nadir KCO as well as nadir KCOc during an active CMV infection and the results obtained before the infection (CMV group). For the control group, Δ KCO and Δ KCOc represent the difference between the highest KCO and KCOc values and the lowest results obtained of the same patients in the study period.

Complement studies

Complement breakdown product of C3: C3d.

Blood was drawn for serial measurements of complement C3d in all patients before transplantation and weekly thereafter throughout the study period. In order to study whether C is activated *in vivo* we performed serial determinations of C3d, a stable breakdown product of C3 (14-16). To block the C-activation *in vitro* for the determination of C3d, blood was collected in iced tubes. While the samples were kept on ice, the blood was allowed to clot for 2 hours before centrifugation in a cooled centrifuge. The samples were subsequently stored at –80°C until the moment when the determinations were made. C3d ($\propto$2D) measurements were made by electroimmunodiffusion, as described elsewhere (16). Anti C3d antiserum was provided by the Red Cross Bloodtransfusion Service, Amsterdam, The Netherlands. Although in the literature $\propto$2D has been equated with C3d, this identification might not be fully correct.

Recently it has been questioned whether C3d is formed in vivo at all (17). By using monoclonal anti C3 antibodies Lachmann et al. suggested that in vivo only C3d,g is formed (17). However, for the sake of convenience, we will designate the activation product in the results obtained as C3d. In our laboratory the normal values for C3d are 0.72-4.68 μg/ml. The intraplate and extraplate variation coefficients were less than 5%. The delta C3d ($\triangle$ C3d) was defined as the actual or percentual increase in C3d levels found during an active CMV infection (as compared to the levels found before the infection: CMV group). For the control group $\triangle$ C3d represented the differences between the highest and lowest C3d levels found in the individual patient during the study period.

Measurements of the biologically active peptide of C activation: anaphylatoxin C3a (measured as C3a des arg).

We were able to perform serial measurements of C3a des arg in 17 patients (8 patients with an active CMV infection and 9 patients of the control group). Blood was drawn for C3a des arg determinations before transplantation and weekly thereafter during the study period. Serial measurements were made of C3a des arg the biologically active fragment that is formed during C-activation (18). Blood was drawn in iced tubes containing 10 mM Na_2 EDTA to block the activation of C *in vitro.* After centrifugation the samples were stored (within 30 minutes of venipuncture) at –80°C until the determinations were made. C3a des arg was measured by radioimmunoassay with ^{125}I C3a des arg (19) using the Upjohn Kit (Upjohn Diagnostics, Kalamazoo MI 49001, USA). The delta C3a des arg was defined as the actual or percentual increase in C3a des arg levels found during an active CMV infection (as compared to the levels found before the infection: CMV group). For the control group $\triangle$ C3a des arg represent the differences between the highest and lowest C3a des arg levels found in the individual patient during the study period.

Statistical analysis

Statistical analysis was performed using the Wilcoxon's rank sum test.

RESULTS

The results of the KCO studies are given in table I. During an active CMV infection KCO and $\triangle$ KCO were significant lower than the results obtained

during allograft rejection or during a stable phase after renal tranplantation. In figure 1, the course of KCO is given of a patient with a CMV infection. While this

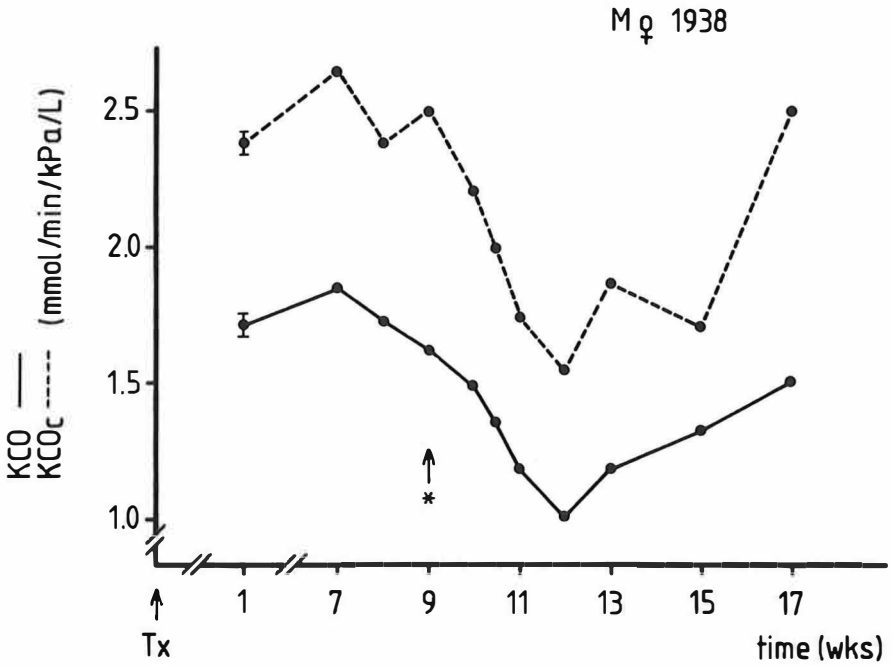

Figure 1. Course of KCO in an asymptomatic patient with a CMV infection.
*: rise in antibodies against CMV EA and LA.
KCO values are means of 3 measurements at the same moment, bars represent SEM.

patient had neither symptoms of the CMV infection nor pulmonary symptoms, it is clearly demonstrated that KCO dropped significantly during CMV infection with and without correction for the hemoglobin concentration. Nadir KCO was significantly lower in the patients with a symptomatic CMV infection than those with an asymptomatic infection ($p < 0.05$). No differences could be demonstrated between patients with a primary infection and those with a reactivation of the virus.

The results of the C3d determinations are given in figure 2. During CMV infection the C3d levels ranged from 3.7-17.1 μg/ml, with a mean value of 7.6 μg/ml and a median of 6.7 μg/ml. In contrast all patients of the control group had C3d levels in the normal range (range 1.0-3.9 μg/ml, mean value 2.6, median 3.2 μg/ml. The difference between the two groups was statistically significant ($p < 0.01$). The Δ C3d in the CMV group ranged from 1.7 to 11.7 μg/ml with a mean value of 5.04 μg/ml (mean 311% increase; range 43-662%). In contrast Δ C3d in the control group ranged from 0.5 to 1.2 μg/ml, with a mean value of 0.83 μg/ml

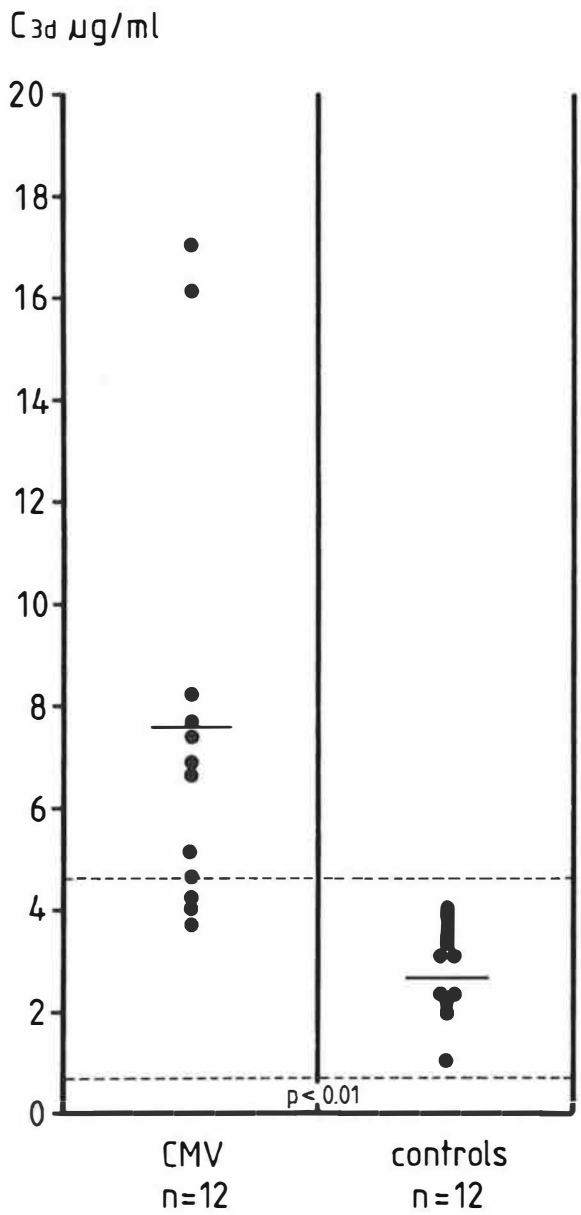

Figure 2. C3d levels of the patients with an active CMV infection and of the control group. Highest values of C3d are given for both groups.

(mean 4.6% increase, range 2-10%). The difference in △ C3d between the 2 groups was statistically significant ($p < 0.01$). No statistical differences could be demonstrated in △ C3d between primary CMV infections and those patients with a reactivation of the virus. Four patients had C3d levels in the normal range during CMV infection (figure 3). But using the patient as his own control there was a rise of 43-650% of the C3d levels during CMV infection of these 4 patients, as compared with the very low levels of C3d in the period before the infection. This may indicate that, although the C3d levels stayed in the normal range, that there was a higher activation grade of the C-system during infection. The results of the determinations of the anaphylatoxin C3a des arg are given in figure 3.

The C3a des arg levels of the patients during an active CMV infection ranged from 229-1000 ng/ml (mean 618, median 462.5 ng/ml). In the control group C3a des arg ranged from 95-225 ng/ml (mean 143, median 142 ng/ml). The difference between the two groups was statistically significant ($p < 0.01$). We designated the maximum level of C3a des arg that could be obtained with this test as 1000 ng/ml, because further dilution of the samples may give unpredictable results (20). The △ C3a des arg in the CMV group ranged from 238 to 798 ng/ml with a mean of 498 ng/ml (mean 259% increase, range 100 to 525%). In contrast △ C3a des arg in the control group ranged from 52 to 100 ng/ml with a mean of 65 ng/ml (mean 32% increase, range 10 to 45%). The difference in △ C3a des arg between the two groups was statistically significant ($p < 0.01$). No statistically significant differences could be demonstrated between primary CMV infections and those patients who had a reactivation of the virus. Although the patients with a symptomatic CMV infection tended to have higher C3a des arg levels than those patients with an asymptomatic infection, this also did not reach the level of statistical significance. An example of the course of C3a des arg together with serial measurements of KCO in a patient with an active CMV infection is depicted in figure 4.

DISCUSSION

Our data are consistent with pulmonary involvement during an active CMV infection after renal transplantation even in absence of pulmonary symptoms, with normal chest radiographs and arterial bloodgas determinations. This may indicate that every patient with an active CMV infection has a subclinical pneumonitis. This agrees with our experience with the so called Bleomycin induced pneumonitis (21) and confirmed our impression that determination of the diffusing capacity of carbon monoxide can be a very sensitive method for the early detection of lung involvement in various conditions.

A rebreathing method for the diffusing capacity was chosen in this study

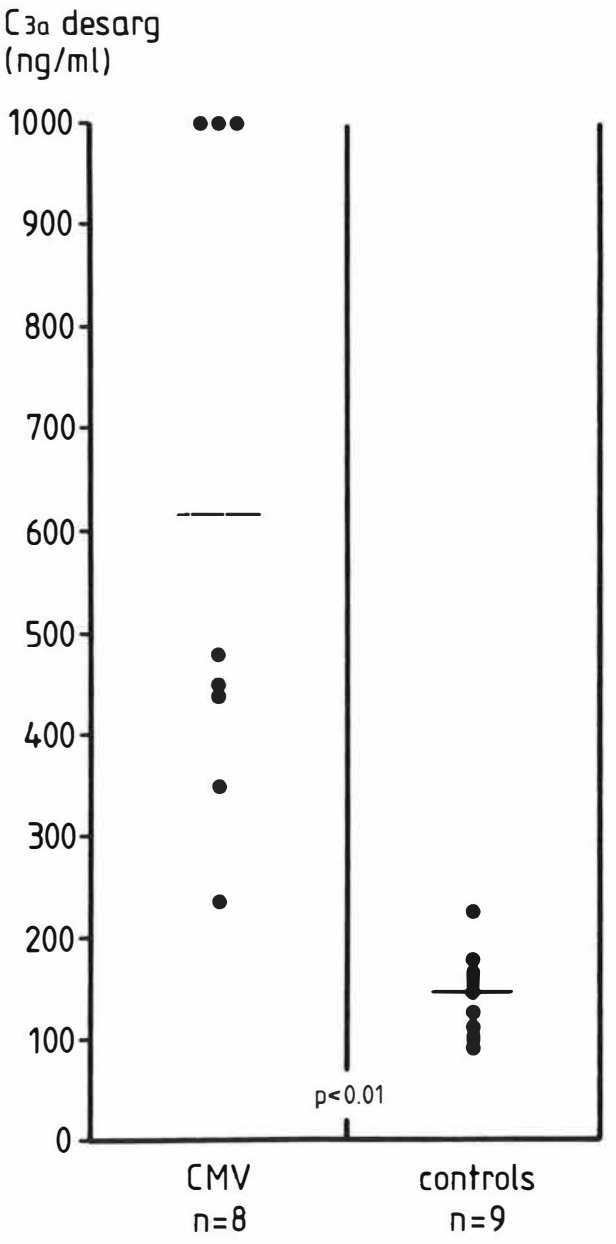

Figure 3. C3a des arg levels in the patients with an active CMV infection and of the control group. Highest values of C3a des arg are given for both groups.

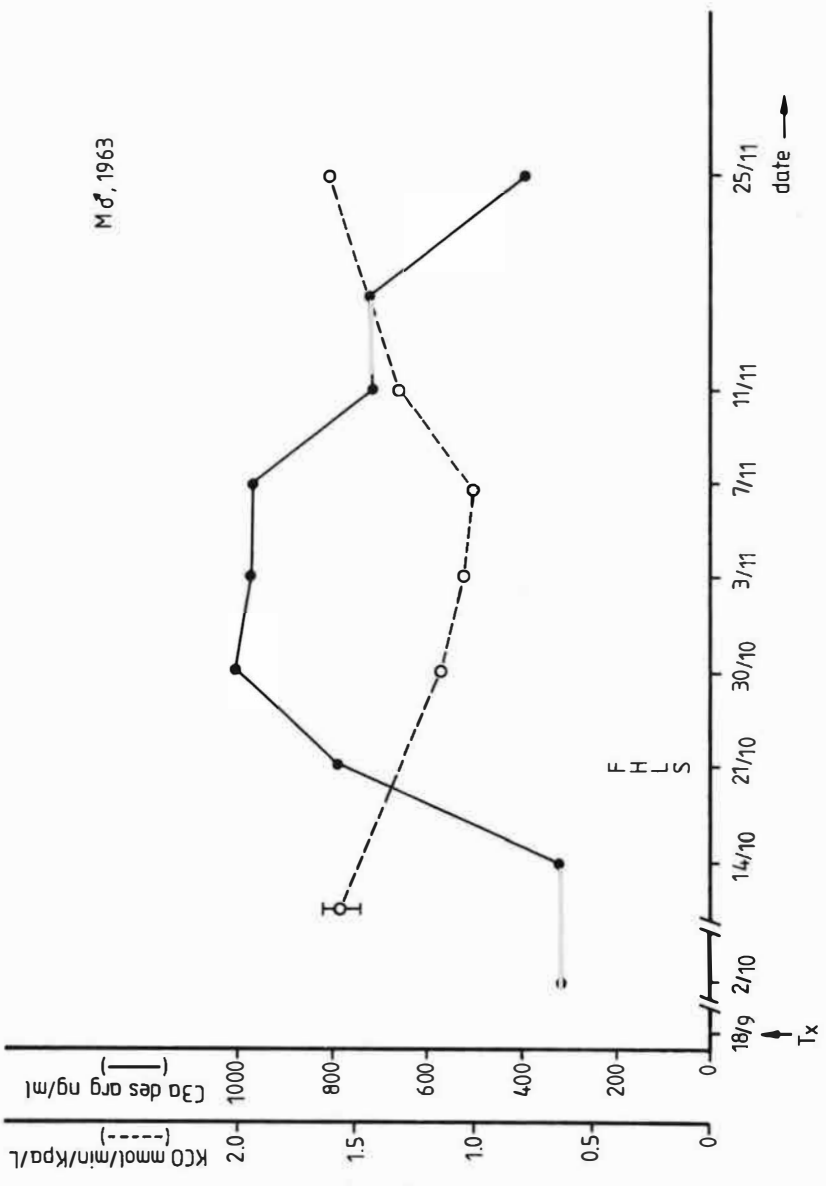

Figure 4. Course of C3a des arg and KCO of a patient with an active CMV infection.
KCO: means of 3 measurements at the same moment, bars represent SEM.
S: moment when CMV could be isolated from the blood followed one week later by seroconversion.
F: Fever.
H: Hematological abnormalities (leucopenia, thrombopenia).
L: Liver function abnormalities.

because some of the patients were bedridden, particularly during the acute phase of the CMV infection. This excluded the use of the single breath method because the bulky equipment needed could not easily be transported to the ward. Furthermore, the use of a spontaneous breathing technique is much easier for bedridden patients. The supine position was chosen in order to standardize our results. Fifteen minutes rest proved to be enough to achieve a stable situation since in our experience the diffusing capacity measured with this rebreathing technique did not change any more after 10 minutes rest in the supine position. We chose a respiration frequency of 30 per minute. For some patients it was practically impossible to achieve frequences of 60 per minute as described by Clark and coworkers (12), and, on the other hand 30 breaths/min is a frequency that is often used in some lung function tests. The results of the diffusing capacity have been expressed as Krogh's coefficient i.e. the specific diffusion, in order to eliminate the influence of the lung volume. The results have been corrected for the hemoglobin concentration according to the equation described in table I. This correction assumes that a linear relationship exists between the hemoglobin content and the diffusing capacity. This is not true for the single breath method according to Cotes (22), Dinakara and coworkers (23), Burgess and colleagues (24) and Riepl (25) between large ranges of hemoglobin concentrations, i.e. 7 to 20 g per 100 ml. However, for a smaller range between 9 and 15 g per 100 ml the curvilinear relationship can be assumed to be a straight line.

Moreover we found evidence of C-activation and formation of anaphylatoxins in these patients during an active CMV infection, and not during allograft rejection or a stable phase after renal transplantation. Two pathways of C-activation have been defined, the classic and the alternative, or properdin, pathway; they share the central C factor C3, after activation of which the final common pathway leads to membrane damage (26,27). After cleavage of C3 the stable conversion product C3d,g is formed (17), the formation of which is a good indicator of C-activation (14,17). During the cleavage process of C3 biologically active polypeptides are also formed and released into the blood stream, commonly denoted as C3a (18). Together with the cleavage products of C5 (C5a), and C4 (C4a), respectively, they are designated as anaphylatoxins (18). They are functionally defined by their actions on the vasculature, smooth muscles, mast cells and certain types of peripheral blood cells (18). Although the physiologic effects of C5a are more impressive than those of C3a, the latter is also capable of acting as immunogen or chemotoxin or can trigger release reactions of mast cells, thereby promoting edema and increasing vascular permeability (18,28). Although results from experimental and clinical studies suggest that the integrity of the C system is important for the defence against micro-organisms and viruses (26,29-31) activation of the C-system could also have detrimental effects (32). A number of investigators have studied the possible role of C in the

immunopathologic aspects of several pulmonary afflictions, as reviewed by Stimler and coworkers (33) and Till and colleagues (34).

In vivo C3a and C5a are rapidly converted to C3a des arg and C5a des arg, respectively, by the anaphylatoxin inactivator carboxypeptidase N (18). Larsen and coworkers (35) and Daniel (36) found that intratracheal instillation of C5a des arg into rabbit lungs produced, to a greater extent than C5a, an acute inflammatory response with a marked neutrophil influx. They concluded that C5a des arg may be the more significant chemical mediator of complement mediated lung injury in vivo (35). The formation of anaphylatoxins promote accumulation of neutrophils and via histamine and probably also non-vasoamine mediators like prostaglandins and platelet activating factor (PAF) they cause smooth muscle contraction and enhance vascular permeability in the pulmonary parenchyma (33). Based on the present available data from clinical and laboratory experiments Stimler and coworkers postulated that C-anaphylatoxins make a significant contribution to the clinical symptoms of numerous airway diseases that show evidence of C-activation or involvement (33). Complement activation and concomitantly changes in DLCO have been demonstrated during hemodialysis treatment (37). Although pulmonary dysfunction during hemodialysis is mainly explained by CO_2 loss via the dialyzer during acetate hemodialysis (causing 'reflex hypoventilation') this might not be the only factor involved. Igarashi and associates (37) could also demonstrate changes in DLCO during *bicarbonate* hemodialysis, where no net CO_2 loss via the dialyzer will occur (37). Moreover they could demonstrate a correlation between the intensity of C activation, the degree of leukopenia and the decrease in pulmonary diffusing capacity (37), suggesting that intrinsic lung dysfunction such as pulmonary leucostasis by the membrane-related activation of C may occur during dialysis (9,37).

In the present study, all patients with an active CMV infection showed evidence of C-activation and formation of anaphylatoxins. The four patients with normal C3d levels during a CMV infection, showed a rise in C3d levels during the CMV infection as compared to the levels before the infection. It is possible that the relatively low levels of C3d before and during the infection were caused by the corticosteroid treatment, as steroids inhibits the C metabolism (38). The concomitant findings of C-activation and formation of anaphylatoxins as well as decreased diffusing capacity for carbon monoxide during an active CMV infection after renal transplantation and *not* during rejection or a stable phase after transplantation may indicate that these findings are linked. From our study it is not possible to elucidate whether the cause of the altered diffusing capacity lies in the membrane or in the blood capillary component, although the similarity with the Bleomycin induced pneumonitis assumes that both components will be altered. Complement activation could for instance have caused the detrimental effect on the KCO we observed during an active CMV infection by promoting

edema (10,18,28,33) in the pulmonary parenchyma (membrane component) or by way of causing micro-aggregates of granulocytes (9,37, 39,40) in the pulmonary microcirculation (capillary component).

In conclusion our data are consistent with pulmonary involvement during a CMV infection after renal transplantation even in asymptomatic patients. Those patients with the greatest drop in KCO might prove to be at greatest risk to develop an overt CMV pneumonitis. Serial measurements of KCO with this simple bedside method can be of help in the sometimes difficult differential diagnosis between CMV infection and allograft rejection. This is illustrated in the patient depicted in figure 5. Although the clinical symptoms mimicked a severe CMV infection (spiking fever, arthralgia, thrombopenia, abnormalities in liver enzymes) it became evident later on that this patient suffered from severe therapy resistant vascular and interstitial allograft rejection, without any laboratory or virological signs of CMV infection. This patient showed no decrease of KCO during the illness at all (fig. 5), in contrast there was a slight rise in KCO during

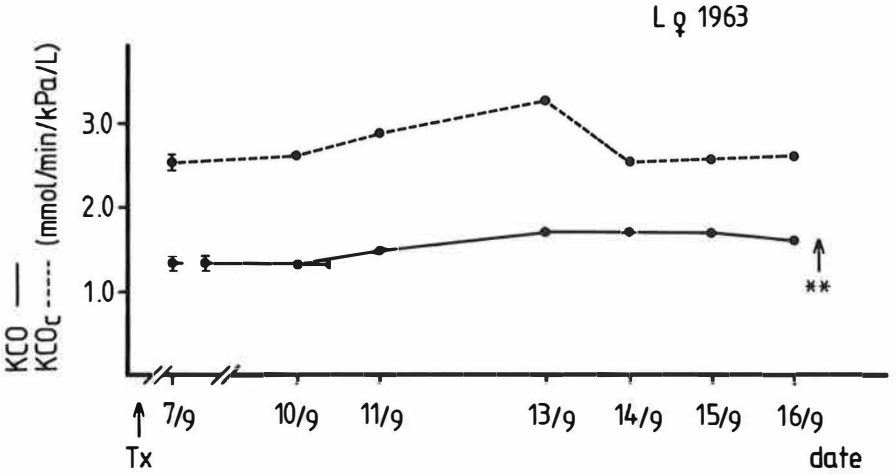

Figure 5. Course of KCO in a patient with severe therapy resistant allograft rejection.
**: transplant nephrectomy.
KCO values are means of 3 measurements at the same moment, bars represent the SEM.

rejection probably due to the fever. The explanation of the decreased KCO during CMV infection might be C-activation we observed during an active CMV infection. Although this explanation is attractive, further studies are warranted to evaluate the exact role of C in the pulmonary events during an active CMV infection after renal transplantation.

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CHAPTER 7

SUMMARY, GENERAL DISCUSSION, AND SUGGESTIONS FOR POSSIBLE FURTHER RESEARCH

CHAPTER 7

SUMMARY, GENERAL DISCUSSION, AND SUGGESTIONS FOR POSSIBLE FURTHER RESEARCH

7.1. **Summary.**

In this thesis studies are presented on systemic effects of cytomegalovirus infection after renal transplantation. Both clinical as well as immunological phenomena are discussed.

In *chapter 1*, sections 1.1.-1.3., fundamental properties of the CMV as well as clinical and immunological features of the CMV syndrome in the immunocompromised host are presented. In *chapter 1*, section 1.4., the aim of this thesis is described. In *chapter 2* a possible new entity of the CMV-syndrome is discussed. Four patients are described with pneumatosis intestinalis associated with an active CMV infection. Since none of the other established etiological features were present, we postulate that the CMV was responsible for the condition. Since vasculitis is one of the suggested etiological factors in the pathogenesis of pneumatosis intestinalis, and vasculitis may be found in the gastrointestinal tract during a severe CMV infection, we suggest the possibility of CMV induced vasculitis as a causal factor in the pathogenesis of pneumatosis intestinalis after renal transplantation. A direct cytopathogenic effect of the CMV on the intestinal mucosa cannot be excluded. Since the condition may be accompanied by free air under the diaphragm, it is important for the clinician to be aware of the possibility of CMV associated pneumatosis intestinalis, in order to avoid unnecessary surgery.

In *chapter 3* the possibility of involvement of the complement (C) system in the defence against CMV is studied. We found that complement is activated during an active CMV infection after renal transplantation and *not* during allograft rejection. Since distinguishing between allograft rejection and cytomegalovirus infection can be very difficult, serial determinations of complement breakdown products might help in patients to differentiate between these two conditions. From this study it was not possible to elucidate whether the classical or the alternative pathway of complement was implicated in the activating process of complement during an active CMV infection. In *chapter 4*, as a consequence of the results presented in chapter 3, we tried to find out whether the classical pathway of C (for instance activated via immune complexes) or the alternative pathway of C is involved in the activation process of C. Serial studies are

presented on the presence of the complement breakdown products C3d, the anaphylactoid factor C3a des arg (the biologically active peptide formed during C-activation), as well as serial studies on alternative pathway involvement in patients with an active CMV infection. Furthermore serial determinations on the presence of circulating immune complexes in these patients are presented. From this study we concluded that, in analogy to the results presented in chapter 3, C is activated during an active CMV infection. But, furthermore we found evidence for the presence of anaphylactoid factors like C3a des arg in the circulation of these patients, that might be responsible for some of the protean manifestations of the CMV-syndrome posttransplant.

Immune complex-like material (both IgM as well as IgG) was also present in the circulation of patients with a primary or secondary CMV infection, but also in some of the patients with allograft rejection. We could not correlate the presence of those complexes with the C-activation found in those patients, suggesting that classical pathway activation via circulating immune complexes was not likely. On the other hand we were able to demonstrate alternative pathway activation in the majority of the patients with an active CMV infection who were tested for alternative pathway involvement. As a consequence of the above mentioned data we postulate that, most likely, the alternative pathway of C is implicated in the activating process of C during an active CMV infection posttransplant. An additional role of direct (without immune complexes) classical pathway activation cannot be excluded.

In *chapter 5* an easy bedside method is described for early detection of pulmonary involvement during an active CMV infection. We were able to demonstrate that *every* patient with an active CMV infection has pulmonary dysfunction (measured as decreased diffusing capacity for carbon monoxide: KCO), even if there are no pulmonary symptoms, a normal chest roentgenogram and a normal arterial blood gas analysis. This may indicate that every patient with a CMV infection after renal transplantation has a *subclinical pneumonitis* possibly evolving to an overt pneumonitis. The patients with a large fall in KCO might prove to be a group of patients particularly at risk. Furthermore, since we did not find any changes in KCO during allograft rejection, serial measurements of KCO might be of help in differentiating between an active CMV infection and rejection after renal transplantation.

The aim of the study presented in *chapter 6* was to elucidate whether the *complement activation* during an active CMV infection (presented in chapter 3 and 4) and the *decreased diffusing capacity for carbon monoxide* found in these patients (presented in chapter 5) are linked. The decreased diffusing capacity for carbon monoxide was found to correlate well with the appearance of the anaphylactoid factor C3a des arg in the circulation of patients with an active CMV infection. Since anaphylactoid factors like C3a des arg and C5a des arg are known

to promote accumulation of neutrophils (which may plug the small vessels of the lung) and furthermore are capable, via histamine and probably also non-vaso-amine mediators like prostaglandins and platelet-activating factor, to cause smooth muscle contraction and enhance vascular permeability and promote formation of toxic O_2 radicals, we postulate that the data presented are in favour of a causal relationship between the pulmonary dysfunction and the C-activation found in patients with a CMV infection posttransplant. Although this explanation is very attractive further studies are warranted to evaluate the exact role of C in the pulmonary events found during an active CMV infection after renal transplantation

7.2. GENERAL DISCUSSION, AND SUGGESTIONS FOR POSSIBLE FURTHER RESEARCH

In order to follow the two major themes presented in this thesis, complement activation and pulmonary dysfunction found during an active CMV infection, one has to address both phenomena together and separately. Complement activation during an active CMV infection is found concomitantly with a decrease in KCO (fig. 1). As presented in chapter 4, complement (C) most likely is activated by

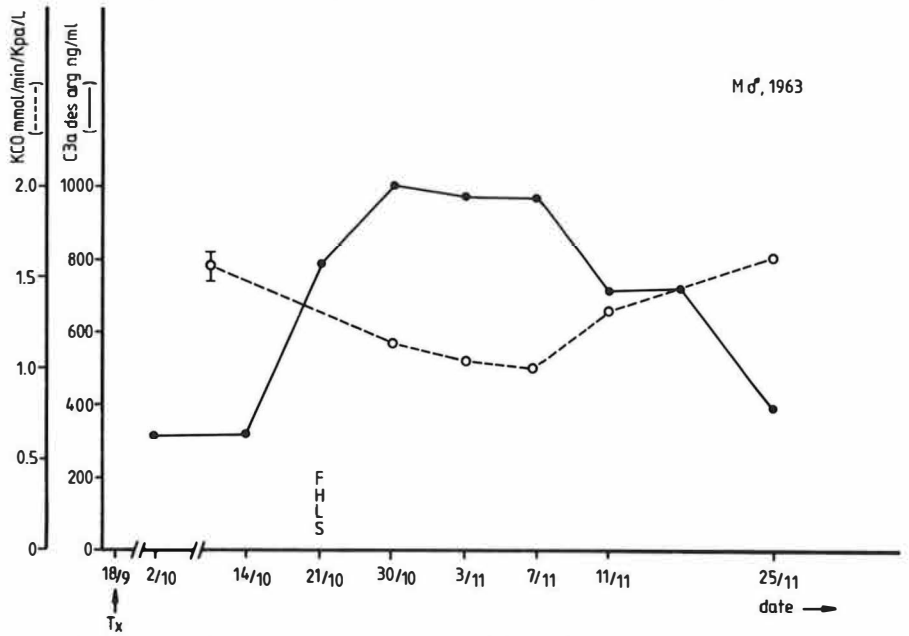

Figure 1. Course of KCO and of C3a des arg in a patient with an active CMV infection.
KCO: mean values are given; bars represent SEM.
F = fever; H = hematologic abnormalities (leucopenia, thrombopenia); L = liver function abnormalities; S = moment when cytomegalovirus could be isolated from the blood followed one week later by seroconversion.

involvement of the alternative pathway. It is not known whether the CMV itself or, alternatively, the CMV infected cells are responsible for initiating the activation process of C. Furthermore it is not known whether the activating process takes place in the circulation (for instance directly by circulating 'free' virus or by circulating cells infected with CMV) or, alternatively, is more confined to organs (for instance the lungs).

Recently van der Bij et al. described a method for rapid and early diagnosis of CMV infection in immunocompromised patients by the detection of CMV specific immediate early antigens (IEA) in peripheral blood leucocytes of these patients (1). In figure 2 the presence of these IEA positive cells is depicted in the

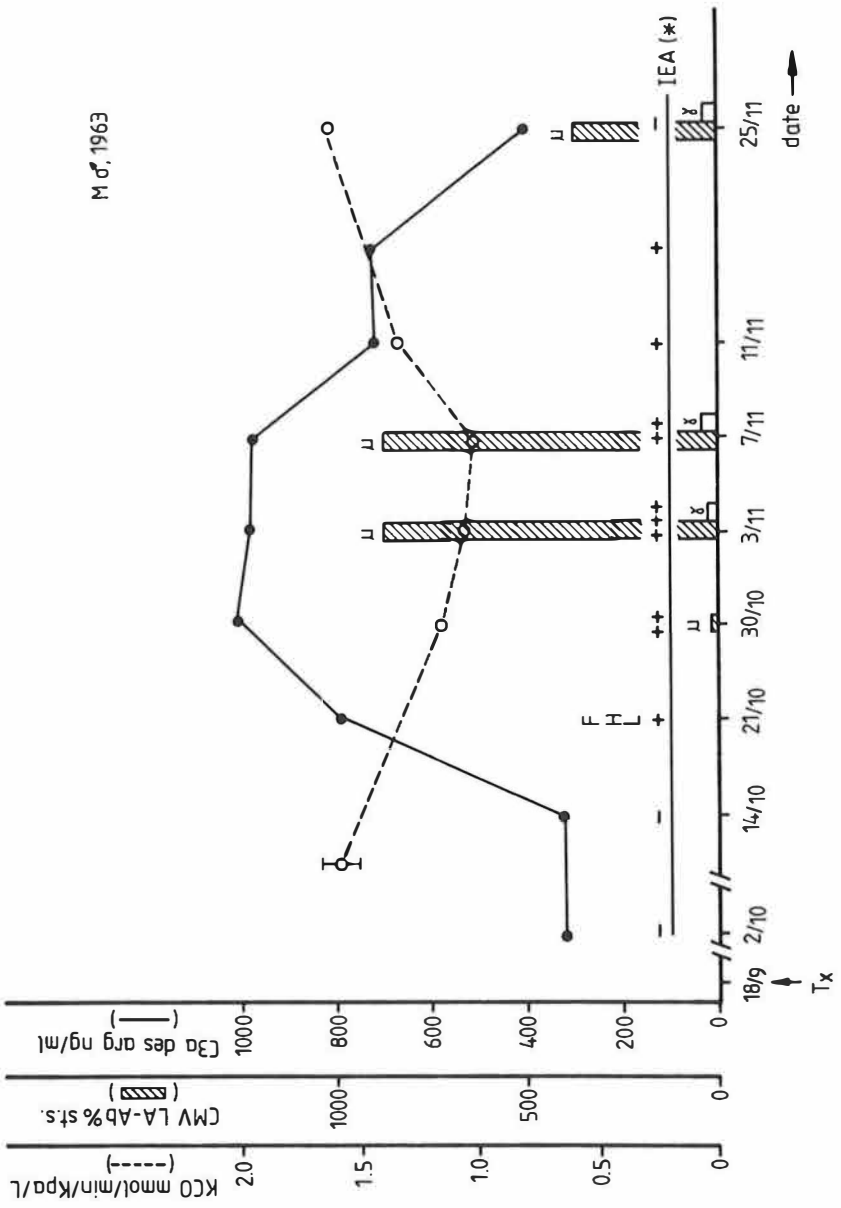

Figure 2. Course of KCO, C3a des arg as well as antibodies against CMV-LA in a patient with an active CMV infection (same patient as shown in fig. 1).
KCO: mean values are given; bars represent SEM.
μ = IgM antibodies against CMV LA.
y = IgG antibodies against CMV LA.
F = fever; H = hematologic abnormatities (leucopenia, thrombopenia); L = liver function abnormalities.
* : IEA = immediate early antigen of CMV present in peripheral blood leucocytes.

same patient as presented in figure 1, together with serial measurements of antibodies directed against CMV LA (Late Antigens) present in the circulation of this patient. The C3a des arg levels as well as the KCO values, shown in figure 1, are also included. From this figure it is clearly demonstrated that C-activation occurred simultaneously with the presence of IEA positive cells and in advance of the appearance of antibodies directed against CMV LA. From these preliminary data presented in figure 2 one can speculate whether the cells expressing immediate early antigens of the CMV might be responsible for the C-activation found in this patient.

The alternative pathway of C involves the interaction of C3, Factor B, and Factor D in the fluid phase leading to formation of small amounts of C3b, some of which may be membrane-bound (2). The fate of this membrane-bound C3b depends on a number of factors including the chemical nature of the surface to which it is attached (2). Interaction between membrane-bound C3b, Factor B and Factor D leads to formation of C3bBb, the C3 convertase (2). This labile complex may be stabilized by properdin (P) leading to enhanced C3 cleavage resulting in full blown amplification of the alternative pathway (2).

Alternatively, an important inhibitor (ß1H) destabilizes the C3bBb complex, and another inhibitor (C3b-inactivator) cleaves and inactivates C3b, thereby impeding the alternative pathway of C (2). The extent of the influence of these two regulatory proteins on the formation and stability of the C3 convertase of the alternative pathway is dependent of the nature of the surface to which C3b is bound (2,3). It has been suggested that membrane bound *sialic acid* modulates the formation and stability of the alternative pathway complement C3 convertase (3,4). Sialic acid (N-acetyl muraminic acid) is a glycoprotein present on certain plasma membranes (5). McSharry et al. found a direct correlation between the amount of sialic acid associated with viral surfaces and its ability to activate the alternative pathway of C (6). They conclude that viruses that *lack* or have *low* amounts of sialic acid on their envelope are efficient activators of the alternative pathway of C, whereas viruses that have *high* amounts of sialic acid on their envelope do not (6). Farrar et al. studied the glycoproteins present on the human cytomegalovirus envelope (7). They demonstrated that the envelope contains sialic acid, although they were not able to quantitate the amount present (7). It is important, in this respect, to stress that cells infected with CMV sometimes change in surface-glycoprotein composition (8), which then may contribute to the modulating process of alternative pathway activation. Whether or not these phenomena play a role in the preliminary data presented in figure 2 is rather speculative, but it could be subject for further research.

In *chapter 5* the way in which complement and factors like C3a des arg and C5a des arg might play a role in the pulmonary dysfunction found during an active CMV infection is discussed. The possible additional role of immune complexes

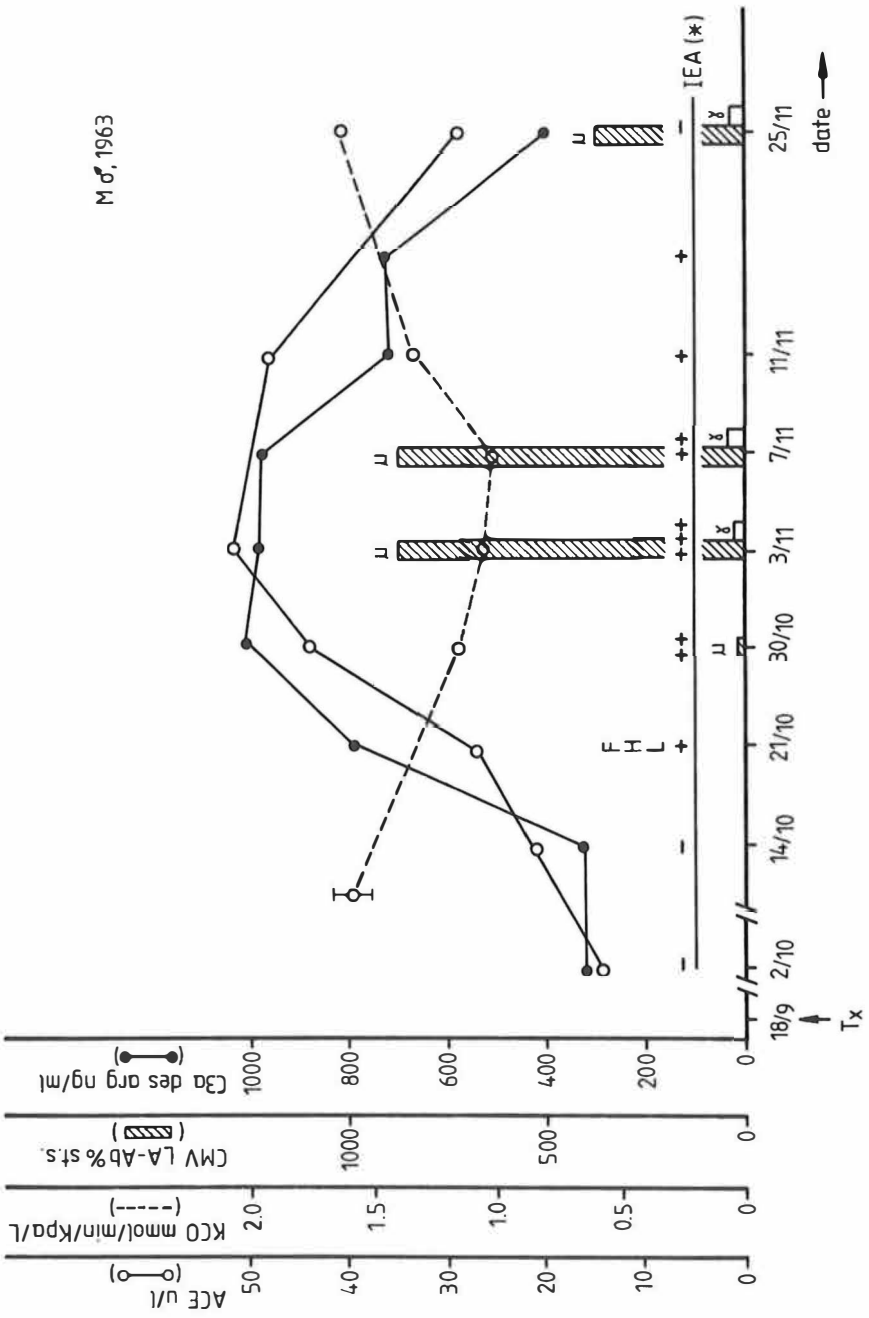

Figure 3. Same patient as shown in figs. 1 and 2. See legends fig. 2.
Course of Angiotensin-Converting Enzyme (ACE) is added to the figure.
Normal values: 0-45 U/l.

(circulating or formed in situ) in the inflammatory process in the lungs during CMV infection is unclear. The endothelium of the alveolar capillary wall is, in contrast to that of the glomerular capillary wall, nonfenestrated. It has been proposed that this type of endothelium prevents easy access of macromolecules such as IgG or immune complexes to the alveolar basement membrane (9). But it is also suggested that if 'factors' increasing the permeability of the alveolar capillary wall are present, they might enhance the accessibility for antibodies to the alveolar basement membrane (9,10,11).

In figure 3 (the same patient as presented in figs. 1 and 2) serial measurements of serum angiotensin-converting enzyme (ACE) are presented together with the data presented in figure 1 and 2. Measurements were carried out by the Central Laboratory for Clinical Chemistry, University Hospital Groningen, The Netherlands (Head Prof.dr. W. van der Slik), using an assay as previously described (12). The course of ACE followed the same curve as the course of C3a des arg in this patient, and correlated well with the decreased KCO found in this patient (fig. 3). Since no other known causes of elevated levels of ACE (such as Besnier-Boeck, hyperthyroidism or diabetes (13)) were present in this particular patient, the elevated levels of ACE found in this patient could indicate endothelial damage (14). One could speculate that the C activation found in this patient might (for instance via the formation of toxic O_2 radicals) be responsible for the endothelial cell damage suggested by the elevated ACE levels found in this patient. Immune complex-like material then could have amplified the inflammatory process in the lungs of the patient. The endothelial wall initially damaged by the sequela of C activation could have become a 'locus minoris resistentiae' so that immune complexes formed during infection (fig. 4) may deposit more easily. This could lead to more C activation and more cell damage, resulting in decreased diffusion of gases across the blood gas barrier. This hypothesis is summarized in figure 5. Pulmonary function tests including the transfer factor for carbon monoxide of the lungs and its two components, the diffusing capacity of the alveolar-capillary membrane and the blood capillary blood volume can be used to test the above mentioned hypothesis.

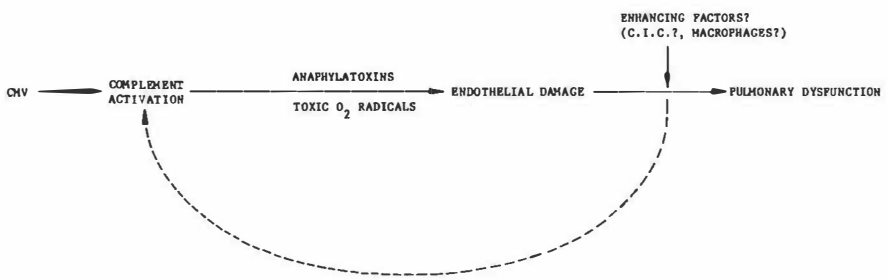

Figure 5. Possible sequential events during a CMV infection leading to pulmonary dysfunction.

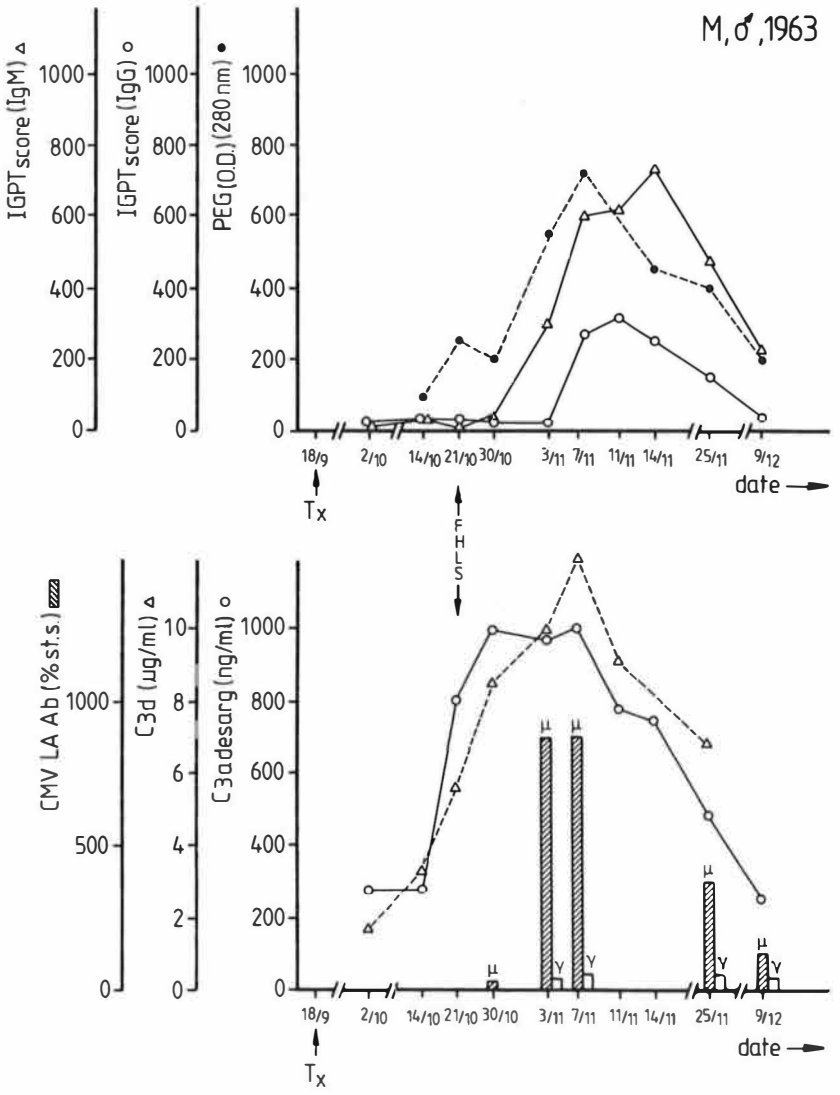

Figure 4. Same patient as shown in figs. 1-3. Course of levels of circulating immune complexes (upper half), levels of C3d as well as C3a des arg (lower half) in a patient with a cytomegalovirus infection.
F = fever; H = hematologic abnormalities (leucopenia, thrombopenia), L = liver function abnormalities, S = moment when cytomegalovirus could be isolated from the blood followed one week later by seroconversion (antibodies against CMV LA).
μ = IgM antibodies against CMV LA.
γ = IgG antibodies against CMV LA.

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SAMENVATTING

In dit proefschrift worden enkele systeem effecten beschreven voorkomend tijdens een cytomegalievirus (CMV) infectie na niertransplantatie. Zowel klinische als immunologische verschijnselen voorkomend gedurende een CMV infectie passeren de revue.

In hoofdstuk 1, sectie 1.1-1.3, worden de fundamentele eigenschappen van het cytomegalievirus beschreven. Verder komen in dit hoofdstuk de klinische verschijnselen aan de orde, welke voor kunnen komen tijdens een CMV infectie.

Een overzicht wordt gegeven van wat er in de literatuur bekend is over de wisselwerking welke bestaat tussen het CMV enerzijds en het immuunapparaat anderzijds bij een patient met verminderde afweer.

De vraagstelling van het onderzoek (sectie 1.4) valt grofweg in te delen in twee afzonderlijke vraagstellingen.

A. De probleemstelling was of serumcomplement (C) geactiveerd wordt tijdens een actief CMV infect na niertransplantatie en of daarbij een onderscheid te maken valt met de situatie voorkomend tijdens afstoting van het transplantaat. Vervolgens werd de vraag gesteld of indien complement inderdaad geactiveerd zou blijken te zijn tijdens een CMV infectie, deze activatie dan zou verlopen via de klassieke activatieroute van C (bijvoorbeeld via circulerende immuuncomplexen) dan wel via de alternatieve activatieroute van C. Samenhangend met de vraag of C geactiveerd wordt is nagegaan of er een samenhang bestaat tussen de bij C-activatie gevormde fysiologische actieve splitsingsprodukten en de bij een CMV infectie zo variërende symptomatologie.

B. De volgende vraagstelling was om na te gaan of door middel van een gevoelige detectiemethode kon worden aangetoond dat *iedere* patient met een actief CMV infect na niertransplantatie *longfunctiestoornissen* heeft, en zo ja of er dan een verband zou kunnen worden aangetoond met de onder A genoemde complementactivatie.

Longfunctiestoornissen ten gevolge van complementactivatie zijn beschreven bij hemodialyse en bij het zogenaamde 'Adult Respiratory Distress Syndrome (ARDS)'. Bij positieve bevindingen in de groep van patienten met een actief CMV infect na niertransplantatie zou dat kunnen inhouden dat mogelijk een soortgelijk pathofysiologisch mechanisme aan deze longfunctiestoornissen ten grondslag ligt als bij beide voornoemde entiteiten.

In hoofdstuk 2 wordt een mogelijk nieuwe ziekte-entiteit toegevoegd aan de vele uitingen van het 'CMV syndroom'. In dit hoofdstuk worden vier patienten beschreven met pneumatosis intestinalis tijdens een actief CMV infect na niertransplantatie. Aangezien geen van de bekende aetiologische factoren welke in verband gebracht zijn met pneumatosis intestinalis aanwezig waren bij deze

vier patienten, wordt gepostuleerd dat CMV verantwoordelijk was voor het ziektebeeld. Aangezien in de literatuur vasculitis als een van de mogelijke aetiologische factoren genoemd wordt, welke ten grondslag kan liggen aan pneumatosis intestinalis, en vasculitis soms gevonden wordt in het maagdarmkanaal van patienten met een CMV infectie, wordt de mogelijkheid van CMV geinduceerde vasculitis geopperd als causale factor in het proces leidend tot pneumatosis intestinalis tijdens een CMV infect na niertransplantatie. Een direct cytopathogeen effect van CMV op de intestinale mucosa kan als oorzakelijke factor niet worden uitgesloten. Aangezien pneumatosis gepaard kan gaan met vrij lucht in de buikholte is het van groot belang voor de klinicus de mogelijkheid van CMV geinduceerde pneumatosis te onderkennen teneinde een onnodige laparotomie te vermijden.

In hoofdstuk 3 wordt ingegaan op de mogelijke rol welke serumcomplement zou kunnen spelen bij een CMV infectie na niertransplantatie.

Complement blijkt geactiveerd te worden tijdens een actief CMV infect en *niet* tijdens afstoting van het transplantaat. Aangezien de differentiaaldiagnose CMV infectie en afstoting soms moeilijk is zou seriële bepaling van complementafbraakprodukten van nut kunnen zijn om te kunnen differentiëren tussen beide diagnoses.

In deze studie was het niet mogelijk om na te gaan of de klassieke dan wel de alternatieve activatieroute van complement betrokken is bij de activatie van complement tijdens een CMV infectie.

In hoofdstuk 4 wordt als een logisch vervolg op de studie vermeld in hoofdstuk 3 nader ingegaan op de wijze waarop C wordt geactiveerd. Longitudinale studies worden gepresenteerd waarbij zowel het voorkomen van het complementafbraakprodukt C3d,g als het anaphylactoide splitsingsprodukt C3a des arg in de circulatie van patienten met een CMV infect wordt bestudeerd.

Bovendien wordt bij een deel van deze patienten de alternatieve activatieroute van C longitudinaal vervolgd.

Voorts wordt met behulp van drie verschillende technieken (Indirecte Granulocyten Fagocytose test, C1q ELISA test en een simpele PEG-precipitatie-test) nagegaan of tijdens een CMV infectie en tijdens rejectie van het transplantaat circulerende immuuncomplexen voorkomen. Uit deze studie mag worden geconcludeerd dat, in overeenstemming met de bevindingen beschreven in hoofdstuk 3, complement geactiveerd wordt tijdens een actief CMV infect en *niet* tijdens afstoting. Bovendien blijkt dat tijdens een CMV infectie biologisch actieve peptiden zoals C3a des arg in de circulatie verschijnen welke mogelijk verantwoordelijk gesteld mogen worden voor enkele van de vele symptomen welke aanwezig kunnen zijn tijdens een CMV infectie.

Immuuncomplexachtig materiaal (zowel IgG als IgM) kon worden aangetoond in de circulatie van vele patienten met een CMV infectie maar ook bij enkele

patienten met een rejectie van het transplantaat. Het moment van verschijnen van deze complexen in de circulatie correleerde *niet* met het tijdstip waarop voor het eerst C activatie kon worden aangetoond, hetgeen activatie van de klassieke route van C via deze complexen onaannemelijk maakt. Daarentegen kon bij de meeste patienten, welke getest werden, een duidelijke activatie van de *alternatieve route* worden aangetoond. Dientengevolge werd geconcludeerd dat waarschijnlijk de alternatieve route betrokken is bij het activatieproces van C tijdens een CMV infect na niertransplantatie.

In hoofdstuk 5 wordt een eenvoudige, goed reproduceerbare en aan het ziekbed uit te voeren gevoelige methode beschreven om longitudinaal longfunctieonderzoek te verrichten bij patienten na niertransplantatie. Met deze methode werd getracht om mogelijke longfunctiestoornissen tijdens een CMV infect op te sporen voordat er klinische symptomen zijn en voordat er röntgenologische afwijkingen te zien zijn op de thoraxfoto.

Aangetoond wordt dat *iedere* patient met een actief CMV infect na niertransplantatie een gestoorde longfunctie heeft (gemeten als verminderde diffusiecapaciteit van de long voor CO:KCO), zelfs indien er geen pulmonale symptomen zijn en met een normale thoraxfoto en een normale arteriële bloedgasanalyse. Dit zou kunnen inhouden dat iedere patient met een CMV infectie een *sub-klinische pneumonitis* heeft, welke in potentie zou kunnen leiden tot een klinisch belangrijke CMV pneumonie.

De patienten met de sterkst gestoorde diffusiecapaciteit zou de groep patienten kunnen zijn welke het grootste risico dragen om een dergelijke ernstige CMV pneumonie te ontwikkelen.

Dergelijke stoornissen in diffusiecapaciteit voor CO werden *niet* gevonden bij patienten met een afstotingsreactie.

In hoofdstuk 6 worden de longfunctiestoornissen, beschreven in hoofdstuk 5, verder geanalyseerd. De verminderde diffusiecapaciteit voor CO vertoonde een goede correlatie met het verschijnen van complementafbraakprodukten zoals C3d,g en C3a des arg in de circulatie van deze patienten.

Aangezien anaphylactoide factoren zoals C3a des arg en C5a des arg accumulatie en klontering van neutrofiele granulocyten kunnen veroorzaken (welke vervolgens kunnen 'pluggen' in de kleine vaten van de long en toxische O_2 radicalen kunnen genereren) en bovendien via histamine en andere mediatoren zoals prostaglandines en plaatjes activerende factor (PAF) vasoconstrictie en oedeem in de vaatwand teweeg brengen, wordt gepostuleerd dat er een causale relatie bestaat tussen de gevonden longfunctiestoornissen en de complementactivatie bij deze patienten. Alhoewel deze theorie aantrekkelijk is, is verder fundamenteel onderzoek noodzakelijk om de exacte rol van complement bij de gestoorde longfunctie tijdens een CMV infectie te duiden.

In hoofdstuk 7 tenslotte worden aan de hand van een theoretische beschouwing enkele suggesties gedaan om het pathofysiologische mechanisme van de longfunctiestoornissen en de complementactivatie nader te bestuderen bij patienten met een actief CMV infect na niertransplantatie.